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PCR-based Methodologies Used to Detect and Differentiate the *Burkholderia pseudomallei* complex: *B. pseudomallei, B. mallei,* and *B. thailandensis*

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Abstract

Methods for the rapid detection and differentiation of the *Burkholderia pseudomallei* complex comprising *B. pseudomallei, B. mallei,* and *B. thailandensis,* have been the topic of recent research due to the high degree of phenotypic and genotypic similarities of these species. *B. pseudomallei* and *B. mallei* are recognized by the CDC as tier 1 select agents. The high mortality rates of glanders and melioidosis, their potential use as bioweapons, and their low infectious dose, necessitate the need for rapid and accurate detection methods. Although *B. thailandensis* is generally avirulent in mammals, this species displays very similar phenotypic characteristics to that of *B. pseudomallei*. Optimal identification of these species remains problematic, due to the difficulty in developing a sensitive, selective, and accurate assay. The development of PCR technologies has revolutionized diagnostic testing and these detection methods have become popular due to their speed, sensitivity, and accuracy. The purpose of this review is to provide a comprehensive overview and evaluation of the advancements in PCR-based detection and differentiation methodologies for the *B. pseudomallei* complex, and examine their potential uses in diagnostic and environmental testing.

Introduction

B. pseudomallei and melioidosis

Melioidosis, a disease caused by the saprophytic gram negative bacillus *Burkholderia pseudomallei*, is endemic in sub-tropical areas such as southeast Asia and northern Australia. The microbe was first described in 1912 by Alfred Whitmore and C.S Krishnaswami as causing a "glanders-like" disease (Whitmore and Krishnaswami, 1912). *B. pseudomallei* was appropriately given the name, "the great mimicker" due to its wide repertoire of clinical manifestations.

The bacterium is commonly found in wet soils and stagnant waters, such as rice paddies, throughout endemic regions. Incidences of disease show a near linear correlation with quantity of rainfall (Currie and Jacups, 2003; Currie et al., 2004). The microbe was isolated in about 25% of soil samples surrounding rice farms in endemic regions of Thailand (Smith et al., 1997). High risk groups involve individuals who are in direct contact with wet soil. They include rice paddy workers, indigenous groups located in southern and eastern Asia, sub-tropical travelers, and individuals who are afflicted with immunosuppressive illnesses, including diabetes mellitus, cirrhosis, thalassemia, renal disease, and alcoholism (Chaowagul et al., 1989; Suputtamongkol et al., 1999; Currie et al., 2000). The disease is acquired through inhalation, contact with cuts/wounds, and occasionally through ingestion of contaminated water. Rare cases of person-to-person transmission have been documented (McCormick et al., 1975; Kunakorn et al., 1991; Abbink et al., 2001; Holland et al., 2002).

In endemic areas, mortality rates are high for melioidosis. Melioidosis had a 39% mortality rate in Singapore in 1996 (Cheng and Currie, 2005), a 19% mortality rate in Australia in 2000 (Currie et al., 2000), a 50% mortality rate in northern Thailand in 2003 (White, 2003), and by 2006, mortality rates were about 40% in northern Thailand (Limmathurotsakul et al., 2010). While melioidosis is beginning to be recognized as an infectious threat (hence a lower mortality rate in recent years), cases of melioidosis are still increasing in number in populated areas such as southern China, Taiwan, Hong Kong, southern India, and Brazil (John et al., 1996; Yang, 2000; Currie et al., 2008; Chen et al., 2010; Brilhante et al., 2012). Eighty percent of children in northern Thailand have developed antibodies against this microbe by the age of four (Kanaphun et al., 1993), however due to the intracellular nature of the infection, antibodies are ineffective. Melioidosis in northern Australia, at the Royal Darwin Hospital, is the most common cause of fatal community-acquired bacteremic pneumonia (Currie et al., 2000) whereas in northeast Thailand, it accounts for 20% of community-acquired bacteremia (Suputtamongkol et al., 1994); being the third most common cause of death by an infectious disease in northeast Thailand (Limmathurotsakul et al., 2010). *B. pseudomallei* isolated from clinical samples accounted for 50% of community-acquired septicemias during the rainy seasons in northeastern Thailand (Chaowagul et al., 1989). Untreated cases of septicemia have mortality rates as high as 80-90% during the first 48 hours of hospital admittance (White et al., 1989; Sanford, 1995). Current cases of melioidosis are probably significantly under-reported due to the lack of diagnostic laboratories in these sub-tropical areas, and misdiagnosis of the disease's non-specific symptoms.

B. mallei and glanders

Glanders, a disease caused by the gram negative bacillus *B. mallei*, is endemic in Africa, Asia, the Middle East and Central and South America (Whitlock et al., 2007). Unlike *B. pseudomallei*, *B. mallei* does not survive well outside of the host. The bacterium primarily infects equine populations

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such as horses, donkeys, and mules. Although equines are the preferred host, infections have been reported in other animals after consumption of glanderous horses (Khan et al., 2012). Equine infection is largely due to ingestion of feed or water contaminated with nasal discharges from infected animals (Whitlock et al., 2007). Human *B. mallei* infection is uncommon, but certain groups are at risk due to exposure to the microbe via infected equines or laboratory cultures. These risk groups include veterinarians, slaughterhouse workers, equine butchers, equine handlers, and laboratory workers. For humans, infection is usually acquired by contact with infectious material through breaks in the skin or mucous membranes such as the eyes, nose, and mouth. Ingestion may not be a common mechanism for *B. mallei* human infection. Consumption of 100 glanderous horses by soldiers (Loeffler, 1886) and even consumption of raw glanderous meat did not produce symptoms of glanders (Gregory and Waag, 2007). However, two individuals did exhibit symptoms of glanders after consumption of milk from a glanderous horse (Loeffler, 1886). The disease has a 95% case fatality rate for untreated septicemia (Spickler, 2008) with death occurring in 7-10 days (Gregory and Waag, 2007), and a 50% case fatality rate in antibiotic-treated patients (Spickler, 2008).

B. thailandensis

B. thailandensis, a saprophytic gram negative bacillus, is readily found in moist soil and stagnant water throughout Southeast Asia and Northern Australia. In 1988, *B. thailandensis* was proposed as a new species from *B. pseudomallei,* because of differences in 16s rRNA sequencing, biochemical profiles, and virulence traits (Brett et al., 1998). Although *B. thailandensis* is considered avirulent for mammals, rare cases of disease have been documented (Dharakul et al., 1999; Lertpatanasuwan et al., 1999; Glass et al., 2006). This BSL-2 bacterium shares several virulence factor homologs with *B. pseudomallei* and *B. mallei*, making *B. thailandensis* the current model organism to study *Burkholderia* pathogenesis. Although *B. thailandensis* is generally avirulent in mammals, the microbe displays very similar characteristics to that of *B. pseudomallei* in most routine diagnostic tests (Thibault et al., 2004) and also co-localizes with *B. pseudomallei* in the environment.

Use as bioweapons

B. pseudomallei and *B. mallei* require BSL-3 management due to their virulence and classification by the CDC as tier 1 select agents. In fact, both pathogens have a history of bioweapon use. In the 1970s, Mao Ze Dong donated a panda bear infected with *B. pseudomallei* to a Paris zoo, and the subsequent infections decimated a large number of zoo animals (Dance and White, 1996). Melioidosis cases have also been reported during the French Indochina War, WWII, and the Vietnam War (Rubin et al., 1963; White, 2003). *B. mallei* was one of the first bioweapons used in the 20th century (Gregory and Waag, 2007) in events such as the American Civil War, World Wars I and II, and the Russian invasion of Afghanistan (Christopher et al., 1997; Alibek and Handelman, 1999; Lehavi et al., 2002).

B. pseudomallei complex

B. pseudomallei and *B. mallei* are known to be phylogenetically similar, and certain isolates of *B. pseudomallei* and *B. mallei* have been shown to differ by a single nucleotide in their 16s rRNA sequences. *B. thailandensis* also shares several characteristics with *B. pseudomallei* and *B. mallei.* Due to these similarities, these organisms are known as the *B. pseudomallei* complex.

Several methods have been developed for detection of various combinations of these species, which include serologic tests (Samosornsuk et al., 1999; Steinmetz et al., 1999; Anuntagool et al., 2000; Chenthamarakshan et al., 2001; Cheng et al., 2006), commercial biochemical tests (Inglis et al., 1998; Lowe et al., 2002; Glass and Popovic, 2005; Amornchai et al., 2007), GC-FAME (Inglis et al., 2003), GLC-FAME (Inglis et al., 2005), microscopic methods (Walsh et al., 1994; Wuthiekanun et al., 2005; Hagen et al., 2011), MALDI-TOF (Inglis et al., 2012), PCR subtractive hybridization technique (Puthucheary et al., 2012), PCR-RFLP (Tanpiboonsak et al., 2004) and gene sequencing (Woo et al., 2002; Gee et al., 2003; Frickmann et al., 2012). Serologic tests may be unreliable in endemic areas due to seroconversion (White, 2003) for those previously exposed to the organism. Therefore, these serologic tests have low sensitivity and specificity in areas of endemicity (Wuthiekanun et al., 2004; Cheng et al., 2006), but may prove useful in non-endemic areas. Biochemical assays have misidentified *B. pseudomallei* as *Pseudomonas spp, B. vietnamiensis, Stenotrophomonas maltophilia,* and *Chromobacterium violaceum* (Inglis et al., 1998; Lowe et al., 2002; Glass and Popovic, 2005). However, many of these methods require culture growth, which may take up to seven days to confirm a diagnosis. Consequently, improperly treated patients may die before receiving a proper diagnosis. Direct testing methods on clinical samples would prove most useful, but due to the low numbers of these organisms in many clinical samples, detection can be difficult.

The high mortality rates of glanders and melioidosis, their potential use as bioweapons, and their low infectious dose necessitate the need for rapid and accurate detection methods. Assays for the rapid detection and differentiation of the *B. pseudomallei* complex have been the topic of much recent research. Optimal identification of these species remains problematic, due to difficulty in developing a sensitive and selective assay. The development of PCR technologies has revolutionized diagnostic testing and these detection methods have been popular due to their speed and accuracy. Therefore, the purpose of this review is to provide a comprehensive overview and evaluation of the advancements in PCR-based detection and differentiation methodologies for the *B. pseudomallei* complex, and examine their potential uses in diagnostic and environmental testing.

Standard detection methods for the B. pseudomallei complex

Isolation and culture of *B. pseudomallei* from bodily fluids of patients remains the "gold standard" in diagnosis of infection. Antibiotic susceptibility and resistance testing, heat resistant alkaline phosphatase tests, oxidation-fermentation reactions of glucose, acid production from maltose, gram stain, and colonial characteristics on differential agar

(Ashdown, 1979; Dance et al., 1989; Hodgson et al., 2009) are tests used in the identification of *B. pseudomallei*.

Isolation of *B. mallei* from the specimen also remains the standard in diagnosis of infection. In addition, the mallein test, and several serologic tests are commonly used to detect glanders in animals, and have varying accuracies and a considerable amount of false positives (Cravitz and Miller, 1950; Neubauer et al., 2005; Naureen et al., 2007; Sprague et al., 2009).

B. thailandensis shares several similar phenotypic characteristics with *B. pseudomallei* that often make these two species difficult to identify in most routine diagnostic tests. However, *B. thailandensis* is biochemically distinguishable from *B. pseudomallei* by its ability to assimilate arabinose as a sole carbon source.

Assay organization

PubMed and Google Scholar databases were searched through December 2012 using various combinations of the following keywords: *B. pseudomallei, B. mallei, B. thailandensis, Burkholderia, Melioidosis, Glanders, PCR, Identification, Detection, Differentiation,* and *Discrimination*. Any of the publications, found from the two databases, that mention other *B. pseudomallei* complex-based assays, were also discussed in this review.

There are many varieties of PCR (i.e., BOX-PCR, PCR-RFLP, MLST PCR, RT-PCR) that are better suited for other applications rather than detection, or require further manipulation of the DNA (i.e., restriction digest, southern blots, or sequencing). Therefore, the PCR methodologies discussed in this review include conventional gel PCR (PCR) as well as quantitative real-time PCR (qPCR)-based methodologies.

The majority of PCR-based studies have validated their sensitivity and specificity by comparison to standard culture techniques. Consequently, the assay sensitivity and specificity values will be determined by comparison to culture in this review. These tests are frequently evaluated on purified DNA (extracted from pure cultures), crude bacterial lysates, or bacterial lysates (some purification), with follow-up studies for the assays' abilities to be used on environmental and clinical samples (direct samples or DNA from direct samples).

Various terms will be used throughout this review that should be defined. Sensitivity describes the number of samples detected by PCR/qPCR relative to culture positive/positive control samples. Specificity describes the number of samples detected by PCR/qPCR relative to culture negative/negative controls samples. Accuracy will denote the combination of sensitivity and specificity. Purified DNA, crude bacterial lysates, bacterial lysates, and clinical and environmental samples are the sample types evaluated by *B. pseudomallei* complex assays. In order to differentiate the sample types, sensitivity and specificity refers to assay accuracy on purified DNA/crude bacterial lysates/bacterial lysates. The addition of "clinical" or "environmental" before sensitivity and specificity values refers to assay accuracy on clinical or environmental samples. The addition of "patient" sensitivity refers to a positive diagnosis of disease from at least one of the melioidosis patients' samples by PCR/qPCR. Some clinical evaluation studies provide accuracies of two different sample types, e.g., clinical sensitivity and patient specificity. Therefore, diagnostic accuracy denotes various combinations of clinical and patient sensitivity or specificity. Some studies evaluate their test(s) for *Burkholderia* on inoculated soil samples or non-inoculated (collected) soil samples. Therefore, two separate environmental accuracies will be reported for these studies. This sample testing method is also observed in clinical studies and therefore, accuracies between inoculated clinical samples and non-inoculated clinical samples will also be reported separately. For the purposes of this review, soil and clinical samples refer to non-inoculated samples unless otherwise specified as "inoculated". In addition, this review will refer to "environmental samples" as any sample type collected from the natural environment.

Three main topics will be discussed in this review, namely, single-species differentiation, multi-species differentiation, and indirect differentiation assays. Single-species differentiation involves assays that claim to identify a single species from the *B. pseudomallei* complex. Multi-species differentiation involves assays that claim to discriminate between more than one species from the *B. pseudomallei* complex. Indirect assays usually involve at least one primer set that is species specific and additional primers to detect a complex of species. When the primers are combined, a unique amplification profile is created and thereby is able to indirectly identify species within the complex.

Table I contains all of the abbreviations that will be used in tables II, III, and IV in alphabetical order. Abbreviations have been listed in table I for each respective column in tables II, III, and IV. Also, species abbreviations were not assigned to individual columns because of their use in seven of the nine categories. Tables II, III, and IV correspond with single-species, multi-species, and indirect differentiation assays, respectively. These tables describe all the testing methods in further detail, which include the tests' authors, species detected, gene target/assay name, the PCR method used, assay sensitivity, assay specificity, the sample information, the species evaluated within the *Burkholderiaceae* family, and the detection limit. On these tables, a black band corresponds to a new assay, and a gray band corresponds to a different detection profile of the *B. pseudomallei* complex. The tests mentioned in the tables are organized in chronological order from electronic publication date and grouped together if the same gene target is used. Footnotes were included in tables II, III, and IV to provide additional insight on particular methods.

A new study is discussed when an author is introduced in the author column of the tables. A blank in the authors column corresponds to the author mentioned previously in the table. Several of the tests have been evaluated/used by other studies. Therefore, an "E" in the species column represents an evaluation study. In tables III and IV, when the "E" is associated in multiple-species/indirect differentiation methods, the sensitivity column will explain which species of the *B. pseudomallei* complex were evaluated. For example if a test detects the *B. pseudomallei* complex, but the evaluation was interested in its ability to detect only *B. pseudomallei*, the accuracy column will denote that only *B. pseudomallei* (Bp) was evaluated. The target/assay and PCR method columns are left blank in evaluation studies since the information is the same as the assay being evaluated.

Table I. Species and Additional Abbreviations used in Tables II, III, & IV

Ba=*Burkholderia andropogonis*	Bk=*Burkholderia kuruiensis*	Bte=*Burkholderia terricola*
Bam=*Burkholderia ambifaria*	Bl=*Burkholderia latens*	Btl=*Burkholderia thailandensis*-like
Ban=*Burkholderia anthina*	Bm=*Burkholderia mallei*	Btu=*Burkholderia tuberum*
Bar=*Burkholderia arboris*	Bml=*Burkholderia mallei*-like	Bu=*Burkholderia ubonensis*
Bc=*Burkholderia cepacia*	Bmu=*Burkholderia multivorans*	Bv=*Burkholderia vietnamiensis*
Bca= *Burkholderia caryophylli*	Bo=*Burkholderia oklahomensis*	Bx=*Burkholderia xenovorans*
Bcal=*Burkholderia caledonica*	Bp=*Burkholderia pseudomallei*	Cm=*Cupriavidus metallidurans*
Bcar=*Burkholderia caribensis*	Bph=*Burkholderia phymatum*	Cn=*Cupriavidus necator*
Bce=*Burkholderia cenocepacia*	Bphe=*Burkholderia phenazinium*	Cp=*Cupriavidus pauculus*
Bcc=*Burkholderia cepacia* complex	Bphy=*Burkholderia phytofirmans*	Cs=*Cupriavidus* species
Bco=*Burkholderia cocovenenans*	Bpl=*Burkholderia plantarii*	Ct=*Cupriavidus taiwanensis*
Bcon=*Burkholderia contaminans*	Bpli=*Burkholderia pseudomallei*-like	Pa=*Pandoraea apista*
Bd=*Burkholderia diffusa*	Bpy=*Burkholderia pyrrocinia*	Pn=*Pandoraea norimbergensis*
Bdo=*Burkholderia dolosa*	Bs=*Burkholderia spinosa*	Pp=*Pandoraea pnomenusa*
Bf=*Burkholderia fungorum*	Bsa=*Burkholderia sacchari*	Ps=*Pandoraea sputorum*
Bg=*Burkholderia gladioli*	Bse=*Burkholderia seminalis*	Rp=*Ralstonia picketti*
Bgl=*Burkholderia glathei*	Bso=*Burkholderia sordidicola*	Rs=*Ralstonia solancearum*
Bglu=*Burkholderia glumae*	Bsp=*Burkholderia* species	Rsp=*Ralstonia* species
Bgr=*Burkholderia graminis*	Bst=*Burkholderia stabilis*	
Bh=*Burkholderia hospita*	Bt=*Burkholderia thailandensis*	

Species Column
E=evaluation
,=denotes differential
[species]=denotes possible species detection
/**=**denotes complex

Target/Assay Column
Chr=chromosome
/=denotes complex

PCR Type Column
M=multiplex
N=nested
O=outer primers
SN=semi-nested

Sample Information Column
CBL=crude bacterial lysates
BL=bacterial lysates
C=culture

Sample Information Column (*cont.*)
CFT=complement fixation test
DFE=DNA from enrichment (selective enrichment duration ranges from a few hours to a few days)
FN=false negative(s)
FP=false positive(s)
PD=purified DNA from isolated culture
+=positive
- **=**negative
[%]=denotes possible accuracy

Detection Limit Column
GE=genomic equivalent
PD=purified DNA from isolated culture
Rxn=reaction

In tables II-IV, a gene target/assay name or PCR method is introduced in their corresponding columns when a different test is being discussed. To differentiate between a target or assay name, the assay name is italicized. When target/assay, PCR method, or species tested columns are blank, they correspond to the test mentioned previously in the table. Any other information left blank in the tables is indicative of information that was not conveyed in the study.

The sensitivity and specificity columns of the tables provide the number of strains tested. If the test is 100% sensitive, the number of strains tested will be listed. If the test displayed less than 100% sensitivity, the column will display the number of strains detected over the total number of positive control strains. Specificity will indicate the number of PCR false positives relative to the total number of negative control strains. In addition, additional accuracy values are included for multiplex assays. The sample information column explains the kind of sample used in the discussed study. For example, "patient (buffy coat DNA)" means patient accuracy was determined from buffy coat samples, and "patient (clinical DNA)" indicates patient accuracy was determined from DNA of various clinical samples. "Patient/clinical DNA" means patient and sample numbers are the same.

Some of these studies have also developed methods to detect other *Burkholderia* species outside the *B. pseudomallei* complex. These tests were briefly mentioned in the review or as a footnote in the tables.

Single-species differentiation assays

B. pseudomallei

The clinical importance of melioidosis has promoted the creation of several assays to detect and identify *B. pseudomallei*.

16s rRNA

A PCR assay targeting the gene for the 16s rRNA (rDNA) was developed for *B. pseudomallei* detection (Brook et al., 1997). The procedure had 100% accuracy, and was ten times more sensitive on the inoculated soil samples than the two culture methods used in the study. This assay also had an environmental sensitivity and specificity of 75% (15/20) and 59.4% (13/32), respectively, for *B. pseudomallei* in soil samples. Due to a low environmental accuracy, the assay appears useful for purified DNA and crude bacterial lysate detection only.

Two qPCR tests based on SYBR Green and TaqMan probes were developed, and both procedures were tested on more than 80 *B. pseudomallei* strains. Both assays reported 100% sensitivity and specificity (Yap et al., 2002). Yap et al recommended the SYBR Green assay because of its lower detection limit compared to the TaqMan procedure.

The 16s rRNA sequences of certain isolates of *B. pseudomallei* and *B. mallei* have been shown to differ by only a single nucleotide, and therefore 16s rRNA assays will likely detect both pathogens as a complex. Although the 16s rRNA assays mention *B. pseudomallei* specificity, many have not been evaluated with *B. mallei*, including the 16s rRNA assays previously described. Consequently, 16s rRNA procedures should not be used in differentiating these two species, but may be suitable for environmental use since *B. mallei* is rarely found in the environment.

Type three secretion system

A PCR test targeting *orf2* within the type three secretion system gene cluster (TTS1) was developed for the purpose of linking virulence with Ara$^-$ *B. pseudomallei* (now known only as *B. pseudomallei*) (Winstanley and Hart, 2000). Although the study was designed to identify virulence, it may also serve as a potential target for *B. pseudomallei* detection. The test had 100% sensitivity and 93.3% (1/15) specificity. The false positive was a *B. thailandensis* strain, which was further tested for possible misclassification by a previously established assay (Dharakul et al., 1999), but was confirmed to be *B. thailandensis*.

Winstanely et al's method was evaluated in three follow-up studies. One follow-up study reported 100% accuracy (Smith-Vaughan et al., 2003). Another follow-up study evaluated the assay's ability to be used on environmental samples (Chen et al., 2002). The procedure had 100% sensitivity on inoculated soil and rice paddy soil samples. Due to *B. cepacia* detection, the test had a specificity of 88.9% (1/9) on inoculated soil samples. On paddy soil samples, the specificity was 0% (5/5). This follow-up study also evaluated the same soil samples against two other PCR studies (Dharakul et al., 1999; Wajanarogana et al., 1999), and both methods also detected *B. pseudomallei* beyond culture (Chen et al., 2002). Consequently, the environmental specificity of Winstanely et al's assay may be higher than the reported 0% for paddy soil samples. The last follow-up study evaluated the test's ability to be used on clinical samples (Gal et al., 2005). The assay had a clinical sensitivity and specificity of 65.4% (17/26) and 95.5% (3/67), respectively, and a patient sensitivity and specificity of 77.7% (7/9) and 100%, respectively. The sample types used affected PCR accuracy. The two false positive clinical samples were sputum samples collected from melioidosis patients undergoing antibiotic treatment. This indicates PCR's sensitivity beyond culture for sputum samples. In addition, a nose swab from a patient with septicemic melioidosis was detected only by PCR. Overall, 9/12 sample types detected by PCR agreed with or went beyond the sensitivity of culture.

Two of the three follow-up studies for Winstanely et al's method reported a sensitivity of 100% on purified DNA and environmental samples. Purified DNA from a *B. thailandensis* strain and a *B. cepacia* inoculated soil sample were detected, and therefore this procedure is not recommended for specific *B. pseudomallei* differentiation. In addition, *B. mallei* strains were not evaluated in any of the studies. There is potential clinical use due to the test's sensitivity beyond culture for sputum samples, but the low sensitivity of the assay would contraindicate its use for diagnosis.

A novel TaqMan qPCR method which targeted *orf2* within the TTS1 was developed, and had a qPCR accuracy of 100% (Novak et al., 2006). The test also had 100% clinical sensitivity on inoculated blood samples.

Novak et al's procedure was evaluated in six follow-up studies. The test's accuracy approached 100% (Trung et al., 2011; Kaestli et al., 2012; Price et al., 2012) with only one strain decreasing the assay's sensitivity. This false negative was a *B. pseudomallei* strain which had a reduced genome that lacked some virulence loci, including *orf2* (Price et al., 2012). Two follow-up studies evaluated the procedure's ability to be used on environmental samples (Kaestli et al., 2007; Trung et al., 2011). Kaestli et al reported 100% specificity on inoculated soil samples. One false negative decreased the assay's sensitivity on soil samples (Trung et al., 2011). This false negative was detected by a different qPCR method (Supaprom et al., 2007; Trung et al., 2011). A total of 10 false positive soil samples were observed by the two environmental follow-up studies (Kaestli et al., 2007; Trung et al., 2011). These false positives agreed with other qPCR tests (Kaestli et al., 2007; Supaprom et al., 2007), indicating Novak et al's qPCR sensitivity beyond culture for soil samples. Overall, the test approached an environmental accuracy of 100%. Three follow-up studies evaluated the assay's ability to detect *B. pseudomallei* in clinical samples (Meumann et al., 2006; Chantratita et al., 2008; Kaestli et al., 2012). The qPCR diagnostic sensitivities were inconsistent. The highest and lowest clinical sensitivity was 80% (Kaestli et al., 2012) and 25.9% (Chantratita et al., 2008), respectively. The highest and lowest patient sensitivity was 90.9% (Meumann et al., 2006) and 33.8% (Chantratita et al., 2008), respectively. Despite the varying clinical sensitivities, the procedure had a diagnostic specificity approaching 100%, as reported in all three clinical follow-up studies.

Novak et al's test appeared useful on purified DNA and environmental samples. The single false negative soil sample was detected by another method (Supaprom et al., 2007; Trung et al., 2011). Therefore, both Novak et al and Trung et al's procedures could be used on environmental samples (Trung et al., 2011). The test had a diagnostic specificity approaching 100%, but the diagnostic sensitivity has varied in subsequent evaluations. In one of the follow-up studies, the assay was able to detect *B. pseudomallei* in all six blood samples from septic shock patients (Meumann et al., 2006). In addition, the test was evaluated with six other qPCR assays on the same clinical samples, and Novak et

Table II. Single-Species Differentiation Assays

Authors	Species	Target/Assay	PCR Method	Sensitivity	Specificity	Sample Information	Species Evaluated	Detection Limit
Brook et al., 1997	Bp	16s rRNA	Gel	100% (6)	100% (13)	PD & CBL	Bp, Ba, Bc, Bca, Bg, Rp, Rs	
	Bp			75.0% (15/20)	59.4% (13/32)	Soil DNA		Ashdown & Galimand-Dodin Enrichment was 10 & 10^4 CFU/mL of inoculated soil, respectively
Yap et al., 2002	Bp	16s rRNA	SYBR Green	100% (>80)	100% (0/23)	PD	Bp, Bc	10 fg (1.5GE)
	Bp	16s rRNA	TaqMan	100% (>80)	100% (0/23)	PD	Bp, Bc	10^2 fg (15GE)
Winstanley and Hart, 2000	Bp	orf2 (TTS1)	Gel	100% (8)	93.3% (1/15)	PD. FP was Bt	Bp, Bt, Bc	
Chen et al., 2002[a]	E			100% (2)	88.9% (1/9)	Inoculated soil DNA. FP was Bc	Bp, Bt, Bc	10 cells/rxn
	E			100% (5)	0% (5/5)	Soil DNA		
Smith-Vaughan et al., 2003	E			100% (116)	100% (19)	PD	Bp, Bt, Bc, Bs, Bv	
Gal et al., 2005	E			65.4% (17/26)	95.5% (3/67)	Clinical DNA. 2 FP were melioidosis patients undergoing treatment while the other FP was a melioidosis patient confirmed via other bodily sites [100% specificity]		
	E			77.7% (7/9)	100% (0/18)	Patient		
Novak et al., 2006[b]	Bp	orf2 (TTS1)	TaqMan	100% (224)	100% (0/139)	PD	Bp, Bm, Bt, Bcc, Bg, Bmu, Bo, Bst, Bv, Cp, Pa, Pn, Pp, Ps, Rsp	76 fg/rxn ($5.2x10^3$ GE/mL)
	Bp			100% (5)		Inoculated blood DNA		~500 cells/mL for 100% detection probability
Meumann et al., 2006	E			73.2% (82/112)	89.2% (31/287)	Clinical DNA		
	E			90.9% (30/33)	94.6% (4/74)	Patient. Detected 6/6 septic shock patients. 4 FP patients did not receive melioidosis treatment and did not develop melioidosis		
Kaestli et al., 2007	E				100% (0/15)	Inoculated soil DNA	Bt, Bcc, Bsp, Cs, Ct	PD: 15 fg (2GE)
	E			100% (13)	92.3% (7/91)	Soil DNA. 6/7 FP were qPCR+ by a wcbG test, but undetected FP was qPCR+ by another sample collected at the same site [100%		Sand/clay: 1 CFU Bp/g soil. Garden/decomposing organic: 1.5 CFU Bp/g soil.

						specificity]		
Chantratita et al., 2008	E			25.9% (30/116)	(99.9% 1/730)	Clinical DNA		
	E			33.8% (26/77)	100% (0/306)	Patient		PD: 20 GE/rxn
Trung et al., 2011[c]	E			100% (29)	100% (0/73)	PD	Bp, Bt, Bc, Bd, Bla, Bpy, Bse, Bv, Rs	3 GE/rxn
	E			96.8% (30/31)	84.2% (3/19)	Soil DNA. 3 FP also detected by Trung et al., 2011's test. [100% specificity] and also detects FN		75 Bp/g soil
Kaestli et al., 2012	E			100% (365)	100% (0/115)	PD	Bp, Bm, Bt, Bc, Bmu, Bo, Bu, Bv, Cs, Rsp	5 GE/rxn
	E			80% (40/50 Bp)	100% (0/22)	Clinical DNA		
Price et al., 2012	E			99.9% (1953/1954)	100% (0/378)	PD. 1 FN due to reduced Bp genome	Bp, Bm, Bt, Btl, Bc, Bce, Bo, Bphy, Bsp, Bu, Bv, Cn, Cs, Rs	
Al-Marzooq et al., 2011	Bp	orf2 (TTS1)	TaqMan Duplex	100% (1)	100% (0/25)	PD	Bp, Bc	14 fg (1/2 duplicates) & 140 fg (2/2 duplicates)
Mustafa et al., 2011	E			100% (1)	88.9% (5/45)	Patient. 5 FP exhibited melioidosis symptoms [100% specificity]		
Neubauer et al., 2007	Bp	mprA	Gel	100% (20)	100% (0/61)	CBL	Bp, Bm, Bt, Bc, Bf, Bv, Rs	10 fg (2GE)
	Bp			100% (2)		Clinical DNA (camel)		
	Bp			100% (1)		Patient (camel)		
Kaestli et al., 2012	E		TaqMan	100% (365)	100% (0/115)	PD	Bp, Bm, Bt, Bc, Bmu, Bo, Bu, Bv, Cs, Rsp	50 GE/rxn
	E			54% (27/50)	100% (0/22)	Clinical DNA		
Suppiah et al., 2010[d] & J. Suppiah, personal communication	Bp	mprA	Gel	100% (66)	100% (0/20)	PD	Bp, Bt, Bc	10 fg/mL
	Bp		SYBR Green (Duplexed w/ Bc test)	100%	100%	PD		
	Bp		Gel	100% (2)	100% (0/16)	Blood DNA		
	Bp			0% (0/2)	100% (0/16)	Serum DNA. Serum results were not c confirmed, but were immunofluorescent antibody confirmed		
Sermswan et al., 1994 & Rattanathongkom et al., 1997[e]	Bp	*pKKU-S23 LPS*	Gel	100% (100)	100% (0/18)	PD & CBL	Bp, Bc	PD: 0.5 fg & BL:1 cell/rxn

	Bp			100% (3/3)	100% (0/5)	Patient/blood DNA		Inoculated blood DNA: 1 cell/mL
Kunakorn et al., 2000	E			31.0% (9/29)	100% (0/17)	Patient/plasma DNA. Some samples collected after treatment.	Bp	
Sermswan et al., 2000	E			95.2% (20/21)	91.7% (9/109)	Patient (blood DNA)		
Sura et al., 1997	Bp	*ME12*	Gel	100% (26)	100% (0/47)	PD	Bp, Bm, Bc, Rp	
Merritt et al., 2006	Bp	phaC	Gel	100% (72)	13% (20/23)	BL. [96.8% (92/95) sensitivity] if used for genus detection	Bp, Bt, Bc, Bmu, Bv	
Supaprom et al., 2007	Bp	Bp loci *8653* & *9438*	TaqMan	100% (17)	100% (0/33)	PD (both tests)	Bp, Bm, Bt, Bc, Bca, Bg, Rp, Rs	50 fg (6 GE) for both tests
	Bp			100% (74)	100% (0/63)	BL (both tests)		
	Bp	*8653*		71.4% (20/28)	82.4% (3/17)	Patient (clinical DNA). 2/3 FP correlated with Thibault et al., 2004's test [94.1% sensitivity]		
	Bp	*9438*		53.6% (15/28)	88.2% (2/17)	Patient (clinical DNA). 2/2 FP correlated with Thibault et al., 2004's test [100% sensitivity]		
Hodgson et al., 2009	E	*8653*		100% (30)	100% (0/13)	PD	Bp, Bt, Bc	
Kaestli et al., 2012	E	*8653*		100% (365)	100% (0/115)	PD	Bp, Bm, Bt, Bc, Bmu, Bo, Bu, Bv, Cs, Rsp	10 GE/rxn
	E			68.0% (34/50)	100% (0/22)	Clinical DNA		
Supaprom et al., 2007 & Trung et al., 2011	Bp	Bp loci	N TaqMan & *8653* test	100% (29)	100% (0/73)	PD	Bp, Bt, Bc, Bd, Bla, Bpy, Bse, Bv, Rs	1 GE/rxn
	Bp			100% (31)	78.9% (4/19)	Soil DNA. 3/4 FP correlated with Novak et al., 2006's test [94.7% specificity]		20 GE/g of soil
Tuanyok et al., 2007	Bp	YLF & BTFC-orf18	M SYBR Green	100% (571)		PD	Bp	
Kaestli et al., 2012	Bp	YLF & BTFC	M TaqMan	99.7% (364/365)	100% (0/115)	PD	Bp, Bm, Bt, Bc, Bmu, Bo, Bu, Bv, Cs, Rsp	5 GE/rxn
	Bp			80% (40/50)	95.5% (1/22)	Clinical DNA. FP is possible contamination		
Andresen et al., 2009	Bp	BPSL1664	SYBR Green	100% (5)	100% (0/18)	PD	Bp, Bm, Bc, Bv, Rp	10 fg (1-2GE)
Badran et al., 2010	E			100% (5)		PD	Bp	10 fg (1-2GE)
Varma-Basil et al., 2004	Bm/[Bp]	16s rRNA	M Molecular Beacons	100% (1)	100% (0/10)	PD	Bm	Bm: ≥50 cells
Scholz et al., 2006	Bm	fliP-IS407 A	Gel	100% (20)	100% (0/57)	CBL	Bp, Bm, Bt, Bc, Bf, Bv, Rs	10 fg (2 GE)

	Bm			100% (1)	0% (2/2)	Patient (horse clinical DNA) 3/3 CFT+ [100% specificity]		
Khaki et al., 2012	E			100% (2)	100% (0/3)	PD	Bm	
Tomaso et al., 2006	Bm	fliP	TaqMan	100% (19)	100% (0/152)	CBL	Bp, Bm, Bt, Bc, Ba, Bam, Ban, Bca, Bcal, Bcar, Bf, Bg, Bgl, Bglu, Bgr, Bh, Bk, Bmu, Bph, Bphe, Bpl, Bpy, Bsa, Bso, Bst, Bte, Btu, Bu, Bv, Pn, Rp	60 fg
	Bm			100% (2)		Patient (horse clinical DNA)		
Schmoock et al., 2009	E					Used to confirm Bm strains		
Ulrich et al., 2006b	Bm	BimA	Gel	93.5% (29/31)	100% (0/56)	PD & 2 FN are avirulent Bm. BL of 1 Bm strain tested with 100% sensitivity	Bp, Bm, Bt, Bc, Bce, Bmu, Bst, Bv	PD: 10 ng & BL: 10^5 CFU/mL
	Bm	ISBma4		100% (31)	66.1% (19/56)	Detected both avirulent Bm strains. FP are Bp & Bt strains		
	Bm	BMAA0610		67.7% (21/31)	33.9% (37/56)	Detected both avirulent Bm strains. FP are Bp & Bt strains		
	Bm	BMAA0611		80.6% (25/31)	42.9% (32/56)	Detected both avirulent Bm strains. FP are Bp & Bt strains		
	Bm	BMA0860		93.5 (29/31)	44.6% (31/56)	Detected both avirulent Bm strains. FP are Bp & Bt strains		
Kaestli et al., 2012	E	BimA				Btl MSMB43 detected		
Ulrich et al., 2006a	Bm	BimA	TaqMan	100% (8)	100% (0/82)	PD (both tests)	Bp, Bm, Bt, Bc, Bmu, Bsp, Bst, Bv	1 pg/rxn (424 GE)
	Bm			100% (3)	100% (0/4)[f]	Patient (clinical DNA of inoculated mice for both tests)		Human blood (for one test): 500 CFU/mL
Khaki et al., 2012	Bm		Gel	100% (2)	100% (0/3)	PD	Bm	
Moore et al., 2004	Bt	AraA-AraB Intergenic Region	Gel	100% (12)	100% (0/12)	PD	Bp, Bt	
Tuanyok et al., 2007	Bt	cheB	SYBR Green	100% (77)	100% (0/8)	PD	Bp, Bm, Bt, Bc	

Table III. Multi-Species Differentiation Assays

Authors	Species	Target/Assay	PCR Method	Sensitivity	Specificity	Sample Information	Species Evaluated	Detection Limit
Lew and Desmarchelier, 1994[g]	Bp/Bm	23s rRNA *PPMA-PPMC*	Gel	100% (41 Bp/3 Bm)	0% (11/11)	PD detected. BL undetected	Bp, Bm, Bc, Bca, Bg, Rp, Rs	
	Bp/Bm	*PPMA-PPM2*				BL undetected		
	Bp/Bm	*PPMB2-PPM2*				BL detected		
	Bp/Bm	*PPMB2-PPM2*		100% (1 Bp)		Direct blood sample (inoculated)		Blood: $1.3x10^4$ cells/mL
	Bp/Bm			100% (2 Bp)		Direct sputum samples (inoculated)		
	Bp/Bm			100% (1 Bp)		Inoculated blood DNA		
	Bp/Bm			100% (2 Bp)		Inoculated sputum DNA		
Brook et al., 1997	E	*PPMB2-PPM2*			84.6% (2/13)	PD & CBL. Bc strains detected	Bp, Ba, Bc, Bca, Bg, Rp, Rs	
Haase et al., 1998	E	*PPMA-PPM2*		20% (2/10 Bp)		Buffy Coat DNA		BL: 1 Bp/rxn. Sputum: 10-30 Bp/rxn. Blood: 100-300 Bp/rxn
Tkachenko et al., 2003 & V. Antonov, personal communication	Bp/Bm	23s rRNA *1s-4as* & *3s-4as*	Gel	100% (49 Bp/12 Bm)	100% (0/55)	PD (both tests)	Bp, Bm, Bml, Bt, Bc	*1s-4as*: 10^3 cells/mL & *3s-4as*: 10^2 cells/mL
	Bp/Bm			100% (8 Bp)	100% (0/11)	Clinical DNA (inoculated hamsters for both tests)		
Antonov et al., 2004	E			100% (15 Bp/14 Bm)	95.0% (2/40)	PD (both tests). 2 FP were Bc	Bp, Bm, Bml, Bt, Bc	Bm BL: 10^2 cells/mL
Kunakorn and Markham, 1995	Bp/Bm	16s-23s rRNA ITS	O Gel	100% (35 Bp)	100% (0/13)	PD	Bp, Bc	
Inglis et al., 2005[h]	E		SN Gel	100% (71 Bp)	100% (0/24)	BL	Bp, Bt, Bc, Bmu, Bv	
Merritt et al., 2006[h&i]	E		SN Gel	98.6% (71/72 Bp)	100% (0/23)	BL	Bp, Bt, Bc, Bmu, Bv	
Couto et al., 2009	E		O Gel	100% (1 Bp)		PD	Bp	
	E			100% (1 Bp)	100% (0/2)	Bronchoalveolar lavage DNA		
Nandagopal et al., 2012	E		O Gel	100% (2 Bp)	100% (0/16)	PD	Bp	<1 CFU/mL
	E				99.7% (1/301)	Buffy coat DNA. Patient of FP sample was successfully treated [100% accuracy]		
Brilhante et al., 2012	E		O Gel	100% (11)		PD	Bp	
Dharakul et al., 1996[j]	Bp/Bm	16s rRNA	N Gel	100% (30 Bp)	100% (0/17)	CBL	Bp, Bc	15 fg (2 GE)
	Bp/Bm			72.7% (8/11 Bp)	100% (0/10)	Septicemic patient/buffy coat DNA		
	Bp/Bm			0% (0/11 Bp)	100% (0/10)	Septicemic patient/plasma DNA		
				66.7% (2/3 Bp)		Localized melioidosis patient/sputum & liver pus		

						DNA		
Haase et al., 1998	E			100% (10 Bp)		Buffy coat DNA		
	E				100% (0/11)	PD	Bc	
	E			96.1% (49/51 Bp)		Clinical DNA. Organism loss of 2 FN [100% sensitivity]		
	E				80% (10/50)	Patient. 2/10 FP patients serologically+ [100% sensitivity and 84.0% specificity]		
Kunakorn et al., 2000	E			41.4% (12/29 Bp)	52.9% (8/17)	Patient/plasma DNA. Some samples collected after treatment		
Tomaso et al., 2004	Bp/Bm	16s rRNA & fliC	TaqMan	100% (64 Bp/9 Bm)	100% (0/179)	PD & CBL (both tests)	Bp, Bm, Bt, Ba, Bam, Ban, Bc, Bca, Bcal, Bcar, Bf, Bg, Bgl, Bglu, Bgr, Bh, Bk, Bmu, Bph, Bphe, Bpl, Bpy, Bsa, Bst, Bte, Btu, Bu, Pn, Rp	Bp PD: 52 GE & 7.5 GE for 16s rRNA & fliC tests, respectively w/ a 95% detection probability
	E					Inoculated blood DNA consistently detected (both tests)		Bp GE: 3000 & 300 for 16s rRNA & fliC tests, respectively
Tomaso et al., 2005[k&s]	E			100% (39 Bp/9 Bm)	100% (0/175)	PD & CBL (both tests)	Bp, Bm, Bt, Ba, Bam, Ban, Bc, Bca, Bcal, Bcar, Bf, Bg, Bgl, Bglu, Bgr, Bh, Bk, Bmu, Bph, Bphe, Bpl, Bpy, Bsa, Bso, Bst, Bte, Btu, Bu, Pn, Rp	PD: 40 GE & Blood DNA: 300 GE/rxn (both tests)
Tomaso et al., 2006	E	fliC		100% (2 Bm)		Patient (horse clinical DNA)		
Chantratita et al., 2007[b&l]	E	16s rRNA		50.9% (59/116 Bp)	99.0% (7/730)	Clinical DNA. 7 FP were non-viable Bp [100% specificity]		20 copies/rxn
	E			61% (47/77 Bp)	100% (0/306)	Patient		
U'Ren et al., 2005	Bp, Bm	P27 SNP	M TaqMan	100% (302 Bp) 100% (37 Bm)	100% (0/53) 100% (0/318)	PD & BL	Bp, Bm, Bt, Bc, Bg, Rsp	10^2 fg (~27 GE)
Bowers et al., 2010	E					2 FP. Bo as Bp and Btl as Bm		
Bowers et al., 2010	Bp, Bm	SNP	M TaqMan	100% (469 Bp) 100% (49 Bm)	100% (0/439) 100% (0/859)	PD	Bp, Bm, Bt, Bc, Bo	10^2 GE
Price et al., 2012	E			100% (1954 Bp)	100% (378)	PD	Bp, Bm, Bt, Btl, Bc, Bce, Bo, Bphy, Bsp, Bu, Bv, Cn, Cs, Rs	
Wongtrakoongate et al., 2007	Bp/[Bm]	BPSL1958	Gel	100% (5 Bp)	100% (5)	PD	Bp, Bt	
Merritt et al., 2006[m]	Bp/Bm	lpxO	Gel	88.9% (64/72 Bp)	100% (23)	BL	Bp, Bt, Bc, Bmu, Bv	

	Bp/Bm		TaqMan	100% (72 Bp)	100% (23)	BL	Bp, Bt, Bc, Bmu, Bv	
Inglis et al., 2008	E		Only TaqMan primers w/ Lab On A Chip	100% (3 Bp)	100% (0/7)	PD	Bp	10 GE
	E					Soil suspensions. 3/5 samples qPCR+ w/ unknown C results		
Kaestli et al., 2012	E		TaqMan	97.7% (365 Bp & 14/23 Bm)	100% (92)	PD	Bp, Bm, Bt, Bc, Bmu, Bo, Bu, Bv, Cs, Rsp	10 GE/rxn
	E			76% (38/50 Bp)	100% (22)	Clinical DNA		
Zhang et al., 2012	Bp	TTS1	TaqMan	100% (13)	100% (0/33)	PD	Bp, Bm, Bt, Ba, Bc, Bpy, Bg	10 fg
	Bm	TFP		100% (11)	100% (0/35)	PD		10 fg
	Bp	TTS1		100% (8)	100% (0/2)	Patient (clinical DNA)		
	Bm	TFP		100% (2)	100% (0/8)	Patient (clinical DNA)		
Rachwal et al., 2012	Bp	Chromosomal target	TaqMan	100% (28 Bp replicates)	100% (0/4)	PD	Bp, Bm	10^2 fg
	Bm			89.3% (25/28 Bm replicates)	100% (0/4)	PD		10^2 fg
	Bp/Bm			100% (28 Bp/28 Bm replicates)	100% (0/3)	PD		10^2 fg
Wajanarogana et al., 1999	Bp, Bt	fliC	Gel	100% (37 Bp) 100% (43 Bt)	100% (0/53) 100% (0/47)	PD	Bp, Bt, Bc	
Chen et al., 2002[a]	E			100% (2 Bp) 100% (1 Bt)	100% (0/9) 100% (0/10)	Inoculated soil DNA	Bp, Bt, Bc	10 cell/rxn
	E			100% (4 Bp)	0% (7/7)	Soil DNA		
Sonthayanon et al., 2002[n]	E			100% (206 Bp) 100% (84 Bt)	100% (0/84) 100% (0/206)	BL	Bp, Bt	20-80 CFU/rxn
	E			100% (4 Bp) 100% (2 Bt)	100% (0/2) 100% (0/4)	Direct soil (inoculated)		200-2000 CFU/rxn
Kao et al., 2003	E					Soil DNA		1 CFU/mL
Chen et al., 2010	E	fliC & 16s rRNA				Soil DNA. 53 samples detected beyond culture		
Price et al., 2012	Bp, Bt/Btl/Bo	SNP in Chr 1 *122018*	TaqMan	100% (1954 Bp) 100% (0/120 Bt/Btl/Bo)	98.4% (6/378) 100% (0/2212 Bt/Btl/Bo)	PD	Bp, Bm, Bt, Btl, Bc, Bce, Bo, Bphy, Bsp, Bu, Bv, Cn, Cs, Rs	Bp: ≥40 fg (5 GE) & Bt: ≥400fg (50 GE)
	Bp, Bt/Btl/Bo	SNP in Chr 2 *266152*		99.9% (1953/1954 Bp) 100% (120 Bt/Btl/Bo)	100% (0/378) 100% (0/2212)	PD. 1 FN is ambiguous		Bp: ≥4 fg (0.5 GE) & Bt: ≥4 ng ($5x10^5$ GE)

Kaestli et al., 2012	E	*266152*		100% (365 Bp)	100% (0/115)	PD	Bp, Bm, Bt, Bc, Bmu, Bo, Bu, Bv, Cs, Rsp	10 GE/Rxn
	E			68% (34/50 Bp)	100% (0/22)	Clinical DNA		
Dharakul et al., 1999	Bp/Bm, Bt	16s rRNA	M N Gel	100% (9 Bp) 100% (5 Bt)	100% (0/22) 100% (0/26)	PD	Bp, Bt, Bc	
	Bp/Bm, Bt			100% (5 Bp)	100% (0/2)	Patient/buffy coat DNA		
Winstanley and Hart, 2000	E					PD. Suspected Bt strain was confirmed as Bt		
Chen et al., 2002[a]	E			100% (2 Bp) 100% (1 Bt)	100% (0/9) 100% (0/10)	Inoculated soil DNA	Bp, Bt, Bc	1 cell/rxn
	E			100% (8 Bp)	0% (2/2)	Soil DNA		
Kao et al., 2003	E					Soil DNA		1 CFU/mL
Chen et al., 2010	E	16s rRNA & fliC				Soil DNA. 53 samples detected beyond culture		
Liu et al., 2002	Bp/Bm/Bt	Repetitive Element	Gel	100% (76 Bp/2 Bm/7 Bt)	100% (0/6)	PD	Bp, Bm, Bt, Bc, Bg, Rp	
Sprague et al., 2002	Bp/Bm, Bt	fliC	Highly Resolving Gel	100% (15 Bp/7 Bm) 100% (30 Bt)	100% (0/46) 100% (0/38)	CBL	Bp, Bm, Bt, Bg, Bv	
Schmoock et al., 2009	E					Used to confirm Bp/Bm strains		
Sprague et al., 2002 & Hagen et al., 2002[o]	Bp/Bm/Bt	fliC	N Gel	100% (3 Bp/1 Bm/1 Bt)	100% (0/13)	CBL	Bp, Bm, Bt, Bc, Bg, Bpl	
				85.7% (6/7 Bp)	100% (0/1)	Inoculated mice/spleen DNA. Actually 100% sensitivity via sequencing		
Ho et al., 2011	Bp/[Bm], Bt	Tat domain & 70 kDa protein	M Gel	100% (43 Bp) 100% (7 Bt)	100% (0/27) 100% (0/63)	PD	Bp, Bt, Bam, Ban, Bar, Bc, Bcc, Bce, Bcon, Bg, Bmu, Bpy, Rp	Triple positive: $<10^3$ copies/rxn
	Bp/[Bm],Bt			100% (1 Bp) 100% (1 Bt)	100% (0/11)	Inoculated sputum DNA (both tests)		Bp: 10^4 CFU/rxn Bt: 10^3 CFU/rxn
	Bp/[Bm],Bt			100% (19 Bp; 4 Bp/Bcc)	100% (0/37)	Soil DNA. Samples confirmed via sequencing		
Koh et al., 2012						Bp test detects Bm		

Table IV. Indirect Differentiation Assays

Authors	Species	Target/Assay	PCR Method	Sensitivity	Specificity	Sample Information	Species Evaluated	Detection Limit
Bauernfeind et al., 1998	Bm	23s rRNA SNP	Gel	100% (3)	100% (0/16)	PD	Bp, Bm, Bc, Bg, Bv, Cn, Rp	
	Bp/Bm			100% (4 Bp/3 Bm)	100% (0/12)	PD		
Antonov et al., 2004	E	Bm test		100% (14)		PD	Bm	BL: 10^3-10^4 cells/mL
Tkachenko et al., 2003 & Antonov et al., 2004[p]	Bm	23s rRNA *Burk 3s-2as* & *Burk 1s-2as*	Non-N Gel	100%(16)	80.0% (11/55)	PD (both tests). FP Bc & Bp strains	Bm, Bp, Bt, Bc	BL: 10^2 cells/mL
	Bp/Bm	*Burk1s-4as* & *Burk3s-2as*		100% (15 Bp/14 Bm)	95.0% (2/40)	PD. 2 FP were Bc strains (both tests)		Bm BL: 10^2 cells/mL
Antonov et al., 2004	Bp/Bm	*B23 s5-a6* & *B23 s7-a8*		100% (15 Bp/14 Bm)	100% (0/40)	PD (both tests)		Bm BL: 10^2 cells/mL
Tkachenko et al., 2003 & Antonov et al., 2004[p]	Bm	*Burk3s-4as* & *Burk 1s-2as*	N Gel	100% (1)		PD. Possible Bc detection	Bm	BL: 10 cells/mL[q]
Antonov et al., 2004	Bp/Bm	*B23 s5-a6* & *B23 s7-a8*	Non-N Gel	100%	100%	Environmental & clinical DNA on both tests (Bm inoculated)		Environmental/Clinical Bm: 10^3 cells/mL
Altukhova et al., 2007	E	*B23 s5-a6*		98.4% (45/46 Bp & 15 Bm)	100% (0/36)	PD	Bp, Bm, Bt, Bc, Bml, Bpli	10^2-10^3 cells/mL
Rainbow et al., 2002	Bp	orf11 (TTS1)	Gel	100% (8 Bp)	100% (0/14)	PD	Bp, Bt	
	Bp	orf7 (TTS1)		100% (8 Bp)	100% (0/14)	PD		
	Bp	bpscJ (TTS1)		100% (8 Bp)	92.9% (1/14)	PD. Faint FP product by Bt E27 strain		
	Bp/Bm	orf13 (TTS1)		100%	100%	PD		
	Bp/Bm/Bt	bpscU2 (TTS2)		100% (3 Bp/3 Bt)		PD		
	Bp/Bm/Bt	SopE homolog (TTS3)		100%	100%	PD		
Rainbow et al., 2002 & Thibault et al., 2004[r]	Bp	orf11 (TTS1)	TaqMan	100% (58 Bp)	100% (0/45)	PD	Bm, Bp, Bt, Ba, Bc, Bca, Bcal, Bcar, Bdo, Bf, Bg, Bgl, Bglu, Bgr, Bmu, Bphe, Bpl, Bpy, Bst, Bv, Pn	Bp: 5 fg DNA/µl
	Bp/Bm	orf13 (TTS1)		100% (58 Bp/16 Bm)	100% (0/29)	PD		
	Bp/Bm/Bt	bpscU2 (TTS2)		100% (58 Bp/16 Bm/3 Bt)	100% (0/26)	PD		
Scholz et al., 2006	E	orf13		0% (0/1 Bm)	100% (0/2)	Patient (horse clinical DNA). 3/3 CFT+ & fliP PCR+ [0%		10 fg (2 GE)

						(0/3) sensitivity & specificity N/A]		
Tomaso et al., 2006	E	orf11		100% (27 Bp)	97.9% (3/144)	CBL. FP were 2 Bt & Bso	Bp, Bm, Bt, Bc, Ba, Bam, Ban, Bca, Bcal, Bcar, Bf, Bg, Bgl, Bglu, Bgr, Bh, Bk, Bmu, Bph, Bphe, Bpl, Bpy, Bsa, Bso, Bst, Bte, Btu, Bu, Bv, Pn, Rp	
	E	orf13		100% (27 Bp/19 Bm)	97.6% (3/125)	CBL. FP were Bt, Bcar, & Bphe		
Supaprom et al., 2007	E	orf11		46.4% (13/28 Bp)	88.2% (2/17)	Patient (clinical DNA). 2 FP correlates with Tomaso et al., 2006's tests [100% specificity]		
Kaestli et al., 2012	E	orf11		100% (365 Bp)	99.1% (1/115)	PD. 1 FP had a late CT for Bm [100% specificity]	Bp, Bm, Bt, Bc, Bmu, Bo, Bu, Bv, Cs, Rsp	10 GE/rxn
	E			70% (35/50 Bp)	100% (22)	Clinical DNA		
Liu et al., 2002 & Lee et al., 2005	Bp/Bm/Bt	Repetitive Element	M Gel	100% (62 Bp/9 Bm/16 Bt)	100% (0/14)	PD & CBL	Bp, Bm, Bt, Bc, Bca, Bg, Rp, Rs	Bp: 10 ng & Bm: 10^2 pg
Lee et al., 2005	Bp/Bt	MprA		100% (62 Bp/16 Bt)	100% (0/23)	PD & CBL		Bt: 10 ng
	Bt	Within repetitive element		100% (16 Bt)	100% (0/85)	PD & CBL		
Scholz et al., 2006	E			0% (0/1 Bm)	100% (0/2)	Patient (horse clinical DNA). 3/3 CFT+ & fliP PCR+ [0% (0/3) sensitivity & specificity N/A]		
Altukhova et al., 2007	Bp/Bm	fliC	Gel	100% (46 Bp/15 Bm)	100% (0/36)	PD	Bp, Bpli, Bm, Bml, Bt, Bc	10^3cells/mL
	Bp/Bm/Bt			100% (46 Bp/15 Bm/5 Bt)	100% (0/31)	PD		10^2-10^3 cells/mL
	Bp/Bm			76% (57/75 Bp)		Clinical acute melioidosis DNA (inoculated animals)		
	Bp/Bm			70% (70/100 Bm)		Clinical acute glanders DNA (inoculated animals)		
	Bp/Bm			33% (33/100 Bp)		Clinical subacute pulmonary melioidosis DNA (inoculated animals)		
	Bp/Bm			37.5% (75/200 Bm)		Clinical subacute glanders DNA (inoculated animals)		
Wattiau et al., 2007	Bp/Bm/ Bt/Bo	narK	Gel	100% (16 Bp/17 Bm/2 Bt)	100% (0/16)	PD	Bp, Bm, Bt, Bam, Ban, Bc, Bce, Bcal, Bdo, Bh, Bmu, Bphe, Bphy, Bpy, Bsp, Bst, Bv, Bx, Cm	

	Bp/Bm/ Bt/Bo	gltB		100% (16 Bp/17 Bm/2 Bt)	12.5% (14/16)	PD		
	Bp, Bm, Bt/Bo	narK & gltB	Molecular Beacon	100% (16 Bp) 100% (17 Bm) 100% (2 Bt)	100%	PD		
Schmoock et al., 2009[s]	Bm	BMAA1051	M Gel	100% (1)	100% (0/13)	PD	Bp, Bm, Bt, Bam, Bc, Bce, Bd, Bf, Bg, Bgl, Bmu, Bst, Bv	
	Bm	BMAA1052		100% (1)	100% (0/13)	PD		
	Bp/Bm	BMAA1916	Gel	100% (1 Bp/1 Bm)[t]	100% (0/12)	PD		
	Bp/Bm	fliC	M Gel	100% (1 Bp/1 Bm)	100% (0/12)	PD		
	Bp/Bt, Bm	BMAA1268		100% (1 Bp/Bt) 100% (1 Bm)	100% (0/12) 100% (0/13)	PD		
	Bp/Bt, Bm	motB		100% (1 Bp/Bt) 100% (1 Bm)	75% (3/12) 76.9% (3/13)	PD		
Koh et al., 2012	Bp	BPSS2022	M Gel	100% (46)	100% (69)	PD & BL	Bp, Bm, Bt, Bc, Rp	Bp: 109ng
	Bp/Bm	BPSL1958		100% (46 Bp/4 Bm)	100% (65)	PD & BL		Bm: 60ng
	Bp/Bm/Bt	BPSS1649		100% (46 Bp/4 Bm/6 Bt)	100% (59)	PD & BL		Bt: 23ng

[a]Author recommends using both Dharakul et al., 1999 & Wajanarogana et al., 1999's assays for improved accuracy of Bp detection
[b]Three hours to complete assay
[c]Novak et al., 2006's assay is more sensitive than culture when combined with Trung et al., 2011's assay on soil samples. Author recommends the use of both Trung et al., 2011 & Novak et al., 2006's assays.
[d]Authors also developed a Bc and genus assay
[e]3.5 hours to complete assay
[f]Clinical sensitivity
[g]Five hours to complete gel assay
[h]Uses three of the four primers from Kunakorn and Markham, 1995's assay
[i]Seven hours to complete assay
[j]Assay completed within a day
[k]Uses forward and reverse primers from Tomaso et al., 2004's assay
[l]Uses forward primer and probe from Tomaso et al., 2004 and Tomaso et al., 2005's assay respectively
[m]2.5 hours to complete assay
[n]Two hours to complete assay
[o]A genus assay was also developed
[p]Burk 1s; 3s; & 4as are primers from Tkachenko et al., 2003's assay
[q]N Burk 1s-2as & B23 s7-a8 assay also had a Bm sensitivity of 10 cells/mL
[r]Uses orf11 forward primer from Rainbow et al., 2002's assay
[s]Also developed a genus assay
[t]Bp product was low

al's method had the highest clinical accuracy (Kaestli et al., 2012). Therefore, Novak et al's qPCR assay is currently the best one available for detecting *B. pseudomallei* in clinical samples.

A novel TaqMan duplex procedure that detects and differentiates *B. pseudomallei* and *S. pneumoniae* was developed (Al-Marzooq et al., 2011). The *B. pseudomallei*-specific test targeting *orf2* within the TTS1 had 100% accuracy. In a follow-up study, Al-Marzooq et al's method was evaluated for its ability to be used on clinical samples from pneumonic patients. This study reported a patient sensitivity and specificity of 100% and 89.1% (5/46) respectively (Mustafa et al., 2011). Three of the five died within 48 hours of hospitalization; a common time frame for melioidosis death. All six melioidosis patients detected by PCR had diabetes and exhibited clinical and radiological symptoms of melioidosis. In addition, a mortality rate of 67% within 48 hours of hospital admittance was also observed. The evidence overwhelmingly indicates the assay's sensitivity beyond culture for pneumonic melioidosis patients.

Al-Marzooq et al's test reported an accuracy of 100% on purified DNA and clinical samples. However, additional *B. pseudomallei* complex strains should be evaluated by the assay. The *orf2* TTS1 has been a heavily evaluated target, showing an accuracy approaching 100%. Therefore, this test will likely have similar accuracies when evaluated. This is the first *orf2* TTS1-targeted procedure that reported higher sensitivity than culture on clinical samples, and it may prove useful for clinical purposes.

Serine metalloprotease

A PCR method which targeted the gene for a serine metalloprotease (*mprA*) had 100% accuracy (Neubauer et al., 2007). The procedure was also tested on a clinical sample from a camel, and the clinical sample was PCR confirmed. In a follow-up study, Neubauer et al's test, adapted for TaqMan use, was evaluated on purified DNA and clinical samples (Kaestli et al., 2012). The assay accuracy was 100%, while the clinical sensitivity and specificity was 54% (27/50) and 100%, respectively.

Neubauer et al's procedure could be used for specific *B. pseudomallei* detection on purified DNA and crude bacterial lysates. The PCR assay, adapted for TaqMan use, and six other qPCR tests were evaluated on the same clinical samples. Neubauer et al's assay had the lowest clinical accuracy (Kaestli et al., 2012). Therefore, other assays may be preferable for clinical detection of *B. pseudomallei*.

Two tests, a PCR and SYBR Green qPCR, were developed to detect and differentiate *B. pseudomallei* by targeting *mprA* (Suppiah et al., 2010). The PCR and qPCR methods were evaluated for their ability to detect *B. pseudomallei*. Both assays had 100% accuracy and the PCR test had 100% clinical accuracy on blood samples. The PCR procedure had a clinical sensitivity and specificity of 0% and 100%, respectively, on serum DNA. As mentioned previously, all assay accuracies are compared to culture confirmed samples. The serum samples were confirmed for the presence of *B. pseudomallei* by an immunofluorescent antibody assay (Vadivelu and Puthucheary, 2000) instead of culture. In addition, other tests were not used to supplement the confirmation of *B. pseudomallei*. Therefore, the PCR clinical accuracy on serum DNA may be inaccurate. Suppiah et al's tests could be used to differentiate *B. pseudomallei* on purified DNA, but *B. mallei* strains were not evaluated. The qPCR method requires further evaluation to determine its ability to detect *B. pseudomallei* in clinical samples. In addition, the PCR method has potential diagnostic use, but needs further evaluation with additional clinical samples.

Additional targets

A novel PCR test based on a DNA probe (Sermswan et al., 1994) had 100% accuracy (Rattanathongkom et al., 1997). This method was also evaluated for its ability to be used on blood samples, and the assay had 100% patient/clinical accuracy (R. Sermswan, personal communication).

Rattanathongkom et al's procedure was used in two follow-up studies that evaluated its ability to detect *B. pseudomallei* in clinical samples of septicemic melioidosis patients (Kunakorn et al., 2000; Sermswan et al., 2000). One follow-up study reported a patient sensitivity of 95.2% (20/21) (Sermswan et al., 2000), while the other reported a diagnostic sensitivity of 31.0% (9/29) (Kunakorn et al., 2000). Overall, nine false positives were reported with a test diagnostic specificity approaching 100%.

Rattanathongkom et al's method appeared useful for *B. pseudomallei* detection using purified DNA and crude bacterial lysates, but the assay needs evaluation with *B. mallei* and *B. thailandensis* strains. The procedure's diagnostic sensitivity is questionable due to conflicting results. Two of the three studies reported a diagnostic sensitivity approaching 100% (Sermswan et al., 1994; Sermswan et al., 2000), while the third study had a clinical sensitivity of 31.0% (Kunakorn et al., 2000). Kunakorn et al's study evaluated the same clinical samples using another PCR method (Dharakul et al., 1996), and Rattanathongkom et al's assay was the least sensitive, but most specific (Kunakorn et al., 2000). Given the conflicting diagnostic assay accuracies, additional clinical evaluation is necessary.

The ME12 PCR procedure had 100% accuracy (Sura et al., 1997). The assay's utility for clinical use is unknown since a southern blot hybridization step was part of this procedure for *B. pseudomallei* detection in clinical samples. Due to the lack of follow-up studies, more recent and better evaluated *B. pseudomallei* differentiation tests may be preferable.

A PCR assay targeting the polyhydroxyalkanoate synthase gene (*phaC*) was developed for the specific differentiation of *B. pseudomallei* (Merritt et al., 2006). The procedure detected all *Burkholderia* species except for three *B. cepacia* strains, indicating a test sensitivity of 100% and specificity of 13.0% (20/23). This assay may be useful for *Burkholderia* genus detection because *phaC* encodes polyhydroxybutyrate synthase, and its accumulation pathway is well conserved within *Burkholderia* (Merritt et al., 2006). If *Burkholderia* genus detection was the object of this study, the assay sensitivity would be 96.8% (92/95). Thus, this PCR test should not be used for the differentiation of *B. pseudomallei* from other species.

Two novel qPCR methods targeting separate *B. pseudomallei* loci were developed, and both tests (9438 and 8653) had 100% accuracy (Supaprom et al., 2007). These procedures were then evaluated for their ability to detect *B. pseudomallei* in clinical samples from septicemic patients. The 8653 test had the higher patient sensitivity of 71.4% (20/28). In addition, 8653 detected all seven fatal septicemias, while 9438 detected only five of the seven fatal

septicemias. Two false positives, detected by both assays, correlated with a previously published test (Thibault et al., 2004), indicating possible sample contamination, and a possible clinical specificity of 100% and 94.1% (1/17) for 9438 and 8653, respectively. In addition, both 9438 and 8653 had a higher clinical sensitivity than Thibault et al's test. The 8653 procedure was evaluated in two follow-up studies that reported 100% accuracy (Hodgson et al., 2009; Kaestli et al., 2012). Therefore, the test appears useful on purified DNA and bacterial lysates. Clinical samples were evaluated using the 8653 method with a reported clinical sensitivity of 68.0% (34/50) and specificity of 100%. When 8653 and six other qPCR tests, which included Thibault et al's assay, were evaluated on the same clinical samples, 8653 and another test were ranked second to last in clinical accuracy. Interestingly, Thibault et al's assay reported a higher clinical accuracy than 8653. The 8653 test may be useful for detection of *B. pseudomallei* in fatal septicemia cases only, and if used, additional supplemental methods should be employed, due to its low diagnostic sensitivity.

A novel nested qPCR method using sequences from Supaprom et al's 8653 test as inner primers was tested for its ability to detect *B. pseudomallei* in purified DNA and soil samples (Trung et al., 2011). The assay had 100% accuracy, and therefore would be useful for differentiation of *B. pseudomallei* using purified DNA, but further evaluation of *B. mallei* strains is necessary. The study also had an environmental accuracy approaching 100%, with four false positives detected. Three of the four false positive soil samples were also detected with Novak et al's TTS1 procedure, indicating that this nested qPCR is sensitive beyond culture on soil samples. Therefore, the nested qPCR and Novak et al's TTS1 methods should be effective if used together for detecting *B. pseudomallei* in soil samples (Trung et al., 2011).

A multiplex SYBR Green qPCR test targeting *Yersinia*-like fimbrial (YLF) and *Burkholderia thailandensis*-like flagellum and chemotaxis (BTFC) gene clusters was developed to type *B. pseudomallei* (Tuanyok et al., 2007). This method had 100% sensitivity, but additional species should be tested since only *B. pseudomallei* strains were evaluated. The same gene targets were also used in a novel TaqMan qPCR procedure designed to detect *B. pseudomallei* in purified DNA and clinical samples (Kaestli et al., 2012). Due to a single false negative, the study had an accuracy short of 100%. The clinical sensitivity and specificity was 80% (40/50) and 95.5% (1/22), respectively. The clinical false negative had a late cycle threshold (C_T) value, indicating possible sample contamination. Kaestli et al evaluated this qPCR method with six other assays for their ability to detect *B. pseudomallei* in clinical samples, and the YLF/BTFC test's clinical accuracy was second best. Therefore, this assay could be used clinically, if Novak et al's TTS1 method is not available.

A SYBR Green qPCR assay which targeted an ATP-binding transport related membrane protein (BPSL1664) was developed, and had 100% accuracy (Andresen et al., 2009). In a follow-up study, the test was evaluated for its ability to detect *B. pseudomallei* signatures in purified DNA obtained from five imported melioidosis travelers (Badran et al., 2010; Christensen, 2013). This study reported 100% sensitivity, but further evaluation of the test is needed for clinical use. Due to a limited sample size, additional bacterial strains, including *B. thailandensis*, should be tested.

Novak et al's TTS1 method has been heavily evaluated on several sample types and has shown consistently better accuracies than other *B. pseudomallei* assays previously discussed. Several clinical evaluations note that the difference in specimen collection methods (with some specimens collected during treatment) may have affected sensitivity. Kaestli et al was able to standardize some of the more heavily evaluated procedures (Thibault et al., 2004; Merritt et al., 2006; Novak et al., 2006; Neubauer et al., 2007; Supaprom et al., 2007; Tuanyok et al., 2007; Price et al., 2012) and showed that Novak et al's assay was the most reliable on clinical samples. Novak et al's test would also be useful in detecting *B. pseudomallei* in purified DNA samples, since all follow-up studies have indicated an accuracy approaching 100%. In addition, Novak et al's and Trung et al's tests should be used together for environmental samples because of the assays' sensitivities beyond culture, which may translate into a more accurate representation of *B. pseudomallei*'s presence in soil.

B. mallei

The historical and potential use of *B. mallei* as a bioweapon reinforces the need for rapid and reliable methods for its detection.

16s rRNA

A multiplex molecular beacon-based procedure, targeting the 16s rRNA, was developed to detect *B. mallei* and three other CDC select agents (Varma-Basil et al., 2004). The test had 100% accuracy, but other *Burkholderia* species, including *B. pseudomallei* and *B. thailandensis* strains, should be tested. This method will likely detect both *B. pseudomallei* and *B. mallei* due to the nearly identical 16s rRNA sequence these pathogens share.

Flagellar biosynthesis protein

A PCR study targeting the flagellar biosynthesis protein insertion element (*fliP*-IS*407* A) was developed to detect *B. mallei*, and reported a test accuracy of 100% (Scholz et al., 2006). The method was clinically evaluated on specimens from three glanderous horses. Seven of the eight specimens were positive in two of the three horses, while the remaining horse was positive in only one of the eight specimens. Overall, the assay had a patient sensitivity of 100% and a specificity of 0% (2/2). All three horses had symptoms of acute glanders, and disease was also confirmed by the complement fixation test (CFT). Therefore, this PCR procedure is likely more sensitive than culture on these types of clinical samples.

In a follow-up study, Scholz et al's test was evaluated for its ability to detect *B. mallei* in purified DNA samples from zoo animals, and the method had 100% accuracy (Khaki et al., 2012). The results also agreed with those of Khaki et al's novel PCR test. Therefore, the *fliP*-IS407 A procedure appears useful on purified DNA and crude bacterial lysates, but further evaluation is needed on clinical samples.

A novel qPCR test targeting the *fliP* gene was developed to detect *B. mallei*, and had 100% assay accuracy (Tomaso et al., 2006). The study was also clinically evaluated on horse samples, and had 100% patient sensitivity. The clinical results also agreed with culture, pathology, and another qPCR method (Tomaso et al., 2004). Therefore,

this assay was useful on both crude bacterial lysates and clinical samples. Tomaso et al's qPCR was also used in another study to confirm the identity of *B. mallei* strains (Schmoock et al., 2009).

All of the *fliP*-based procedures had 100% accuracy on purified DNA or crude bacterial lysates, and therefore would be useful for *B. mallei* detection. Although the *fliP*-based studies had 100% diagnostic sensitivity, the limited clinical sample sizes used mandate further evaluation with larger numbers of samples.

Burkholderia intracellular motility A

Five PCR assays were developed to detect *B. mallei*, and only the test targeting the *Burkholderia* intracellular motility A gene (*BimA*) reported the highest accuracy. This *BimA* procedure reported a PCR sensitivity of 93.5% (29/31) and specificity of 100% (Ulrich et al., 2006b). The two false negatives were avirulent *B. mallei* strains containing a reduced genome lacking some genes including *bimA*. The N-terminus region of *BimA* was used to develop two novel qPCR procedures which had 100% accuracy (Ulrich et al., 2006a). Both tests were evaluated for their ability to detect *B. mallei* in clinical samples, and both had a qPCR patient accuracy of 100%. Results of this study showed that lung samples had the highest level of clinical sensitivity, while blood samples had the lowest level of clinical sensitivity.

The *BimA* method had a *B. mallei* sensitivity approaching 100%. The two false negatives, detected by the *BimA* PCR assay, were avirulent *B. mallei* strains. The avirulent strains previously described were not tested on the two *BimA* qPCR assays, and therefore *BimA* assays may only detect virulent *B. mallei.* The qPCR assays had a patient accuracy of 100%, but additional clinical evaluation would be useful because of a limited sample size.

Another potential problem with the *BimA* assays is that *B. pseudomallei* strains from Australia also contain the N-terminus region of *BimA*, indicating that the *BimA* tests may not be suitable for specific *B. mallei* differentiation from Australian *B. pseudomallei* isolates (Sitthidet et al., 2008). In addition, *B. thailandensis*-like strain MSMB43 was detected by the *BimA* PCR assay (Kaestli et al., 2012).

Additional targets

Khaki et al developed a novel PCR test for *B. mallei* detection which had 100% assay accuracy (Khaki et al., 2012). The method was also evaluated for its ability to detect *B. mallei* on purified DNA samples from zoo animals. The method had 100% accuracy, similar to the results of Scholz et al's *fliP* assay previously described. Therefore, this procedure has potential for use on purified DNA, but needs further evaluation using additional *Burkholderia* strains, including *B. pseudomallei* and *B. thailandensis*. This method also requires clinical evaluation.

B. thailandensis

Since *B. thailandensis* is not a select agent and rarely causes human disease, only two methods exist that specifically differentiate *B. thailandensis*: a PCR assay targeting arabinose assimilation genes (Moore et al., 2004) and a SYBR Green qPCR test targeting chemotaxis receptor methylesterase (*cheB*) (Tuanyok et al., 2007). Both had 100% accuracy and would be useful on purified DNA, but Moore et al's method needs evaluation with additional *B. pseudomallei* and *B. mallei* strains.

Multi-species differentiations assays

The genomic sequence similarity of members of the *B. pseudomallei* complex has promoted the creation of several assays to detect and differentiate various combinations of these species. A slash between *Burkholderia* species will denote a complex, i.e., *B. pseudomallei/B. mallei*.

B. pseudomallei and B. mallei

The significance of the etiologic agents of melioidosis and glanders, with regards to their clinical characteristics and potential use as bioweapons, has promoted the creation of several tests to specifically detect these pathogens in assays that may or may not differentiate between species.

23s rRNA

Three PCR methods targeting the 23s rRNA were developed for detection of *B. pseudomallei/B. mallei* (*Bp/Bm*) (Lew and Desmarchelier, 1994). One test (PPMA-PPMC) was unable to detect *Bp/Bm* in bacterial lysates, but reported 100% accuracy on purified DNA. The other two tests were evaluated for their ability to detect *Bp/Bm* in bacterial lysates, and only one assay (PPMB2-PPM2) detected the pathogens. The PPMB2-PPM2 test was also evaluated for clinical use and had 100% clinical sensitivity.

Two follow-up studies evaluated the 23s rRNA assays for their ability to detect *Bp/Bm* in purified DNA and clinical samples. Although Lew and Desmarchelier's PPMB2-PPM2 method had showed 100% clinical accuracy, one of the follow-up studies indicated this procedure also detected *B. cepacia* strains, and therefore had a PCR accuracy less than 100% (Brook et al., 1997). This assay should be evaluated on *B. cepacia*-inoculated clinical samples to determine clinical accuracy. The second follow-up study using the PPMA-PPM2 procedure had a clinical sensitivity of 20% (2/10) on buffy coat samples (Haase et al., 1998). These buffy coat samples were also tested with another PCR method (Dharakul et al., 1996) which was 100 times more sensitive than PPMA-PPM2 (Haase et al., 1998). These 23s rRNA methods may not be suitable for purified DNA, crude bacterial lysates, and clinical samples due to *B. cepacia* detection and low sensitivity. Evaluation of additional *Burkholderia* species (including *B. thailandensis*) and clinical samples, may be useful for the other test in Lew and Desmarchelier's study (PPMA-PPMC). However, more recent and better evaluated *B. pseudomallei* differentiation tests are preferable.

Two novel PCR assays were developed that targeted the 23s rRNA to detect *Bp/Bm*, and had 100% accuracy (Tkachenko et al., 2003). These tests were clinically evaluated on *B. pseudomallei*-inoculated hamster samples, and both procedures had 100% clinical sensitivity. In a follow-up study, both procedures detected two *B. cepacia* strains, and therefore, the PCR accuracies are less than 100% (Antonov et al., 2004). The tests should not be used on purified DNA. Although the clinical specificities of the tests were 100%, the assays are likely to detect *B. cepacia*, and therefore, these should not be used on clinical samples.

Tkachenko et al's two assays were used as components of a novel nested PCR assay to increase detection sensitivity

of *Bp*/*Bm* (Antonov et al., 2004), which will be discussed in the indirect assay differentiation section.

16-23s rRNA internal transcribed spacers

Four primers, targeting the 16-23s rRNA internal transcribed spacers (ITS), were developed and used in various combinations in PCR assays to detect *Bp/Bm* (Kunakorn and Markham, 1995). The non-nested (outer primers) PCR assay had 100% accuracy, but *B. mallei* and *B. thailandensis* strains were not evaluated. The other PCR tests were coupled with solution hybridization in the study, and therefore, accuracies of these methods are unknown.

Five subtly modified follow-up studies tested the 16-23s rRNA ITS tests for their ability to detect *Bp/Bm* in purified DNA. These subtle modifications use the same primer sequences specified in the 16-23s rRNA ITS assay, and consequently, the follow-up studies are not different enough to be considered new tests.

Two follow-up studies used the semi-nested method to detect *Bp/Bm*, and reported an accuracy approaching 100% (Inglis et al., 2005; Merritt et al., 2006). The remaining three follow-up studies used the non-nested procedure to detect *Bp/Bm*, and reported accuracies of 100% (Couto et al., 2009; Brilhante et al., 2012; Nandagopal et al., 2012). Two non-nested follow-up studies tested the 16-23s rRNA ITS assays for their ability to detect *Bp/Bm* in clinical samples, and reported clinical accuracies approaching 100% (Couto et al., 2009; Nandagopal et al., 2012). In addition, a false negative buffy coat sample was detected, and the patient associated with this sample was successfully treated for melioidosis (Nandagopal et al., 2012). Therefore, the non-nested assay has possible *Bp/Bm* detection sensitivity beyond culture for buffy coat samples.

It appears that the 16-23s rRNA ITS studies, semi-nested and non-nested, were useful for the detection of *Bp/Bm* in purified DNA or bacterial lysates. Both assay types may be useful for *Bp/Bm* detection, but require further evaluation with additional bacterial strains including *B. mallei* and *B. thailandensis*. The non-nested procedure could be useful clinically, but needs further evaluation on additional clinical samples. The semi-nested method also requires evaluation for clinical use.

16s rRNA

A nested PCR method to detect *Bp/Bm,* targeting the 16s rRNA, reported an accuracy of 100% (Dharakul et al., 1996). Various clinical samples were also evaluated by the test to explore its ability to detect *B. pseudomallei* from septicemic patients. The assay had a sensitivity and specificity of 36.4% (8/22) and 100%, respectively, on clinical samples from septicemic patients. If accuracies were specified by sample type, the PCR diagnostic sensitivities would be 72.7% (8/11) and 0% (0/11) for buffy coat and plasma samples, respectively, for melioidosis septicemic detection. In addition, the test has a clinical and patient sensitivity of 66.7% (2/3) for identification of localized melioidosis. Interestingly, of the eight blood-culture-negative sputum samples from localized melioidosis patients, one patient's sputum sample was detected by this PCR assay.

A follow-up study of Dharakul et al's procedure reported 100% specificity (Haase et al., 1998), but two follow-up clinical studies reported inconsistent diagnostic PCR accuracies (Haase et al., 1998; Kunakorn et al., 2000). One clinical follow-up study reported a clinical accuracy less than 55% on plasma samples (Kunakorn et al., 2000). Another clinical follow-up study reported 100% assay sensitivity on buffy coat samples (Haase et al., 1998). The two false negatives and 10 false positives reported in this same study shed doubt on the assay's diagnostic accuracy (Haase et al., 1998). These two false negatives were undetected because of the age of one of the samples, and centrifugation of the other sample which resulted in organism loss prior to testing. Of the ten patients with false positive clinical samples, two were serologically positive, suggesting previous exposure to the organism. Therefore, the possible patient test sensitivity and specificity is 100% and 84% (8/50), respectively. In addition, three serologically-positive but culture-negative blood specimens from melioidosis patients were confirmed positive for *Bp/Bm* by PCR. These patients also responded positively to treatment.

This method may be used for *Bp/Bm* detection on purified DNA and crude bacterial lysates, but the procedure needs further evaluation with *B. mallei* and *B. thailandensis* strains. The low diagnostic sensitivity of Dharakul et al's assay on one of the two clinical follow-up studies could be attributed to some samples being collected during treatment, and/or differences in specimen collection procedures (Kunakorn et al., 2000). The same plasma samples from the clinical follow-up study were also evaluated for *Bp/Bm* using another PCR test (Rattanathongkom et al., 1997) in which Dharakul et al's test was the highest in sensitivity, but lowest in specificity (Kunakorn et al., 2000). In addition, the follow-up study on inoculated buffy coat samples showed the assay to be 100 times more sensitive in detecting *Bp/Bm* than a previously described 23s rRNA method (Lew and Desmarchelier, 1994). The variability in the assay's diagnostic accuracies in the clinical studies may have been due to the sample type. The higher diagnostic accuracy reported came from the follow-up study evaluating buffy coat samples (Dharakul et al., 1996; Haase et al., 1998), and the lower diagnostic accuracy reported came from the follow-up study evaluating plasma samples (Kunakorn et al., 2000). Despite the conflicting data, the 16s rRNA procedure has potential diagnostic use in detecting *Bp/Bm*, but additional clinical evaluations should be performed.

16s rRNA and flagellar structural protein

Two qPCR assays were developed targeting the 16s rRNA and flagellar filament structural protein (*fliC*) for *Bp/Bm* detection (Tomaso et al., 2004). The two procedures performed equally well on purified DNA and crude bacterial lysates, with 100% accuracy for *Bp/Bm* detection. In the same study, inoculated blood samples were consistently detected by both tests.

Two studies (Tomaso et al., 2005; Chantratita et al., 2007) used different combinations of the primers and probe from Tomaso et al's methods. Therefore, the original assay and the tests used in the follow-up studies are all slightly different from each other. They could be considered as novel procedures, but due to the similarities of the primers, they are best classified as follow-up studies.

One follow-up study using the 16s rRNA and *fliC* assays had 100% accuracy in detecting *Bp/Bm* (Tomaso et al., 2005). The same study also reported an assay targeting the ribosomal protein subunit 21 (*rpsU* 21), which likely identifies the *Burkholderia* genus (Hagen et al., 2002).

Another follow-up study explored the ability of the original *fliC* assay (H. Tomaso, personal communication) to detect *B. mallei* in clinical samples from two horses suspected of glanders, and the assays had 100% patient sensitivity, which correlated with a *fliP* study previously described (Tomaso et al., 2006). A third follow-up study evaluated the ability of the 16s rRNA assay to detect *Bp/Bm* in clinical samples. This test had a clinical sensitivity and specificity of 50.9% (59/116) and 99.0% (7/730), respectively (Chantratita et al., 2007). These data corresponded to a qPCR patient sensitivity and specificity of 61% (47/77) and 100%, respectively. Depending on the sample type, varied levels of clinical sensitivity were observed as seen by the difference between the lowest level of clinical sensitivity found in blood (25%) and the highest level of clinical sensitivity found in pus/fluid specimens (85.7%). Interestingly, the qPCR and culture-confirmed patients had a 100% mortality rate, suggesting the assay can be used for faster diagnosis of potentially fatal melioidosis cases. In addition, the seven false positive clinical samples detected non-viable *B. pseudomallei* from confirmed melioidosis patients (culture confirmed from different body site collections). Therefore, this method has a possible clinical specificity of 100%.

The original 16s rRNA and *fliC* procedures and their subtle variation counterparts appear useful in detecting *Bp/Bm* in purified DNA samples and crude bacterial lysates (Tomaso et al., 2004; Tomaso et al., 2005). The original *fliC* method appears useful clinically, but requires further evaluation of additional clinical samples since only two glanderous horses were evaluated. The other follow-up study used only the 16s rRNA test, and had a patient sensitivity and specificity of 61% and 100%, respectively (Chantratita et al., 2007). Although patient sensitivity was low, the assay was able to detect all fatal cases of melioidosis. Furthermore, higher sensitivity yields are possible if the optimum specimen type is used, such as sputum or pus, and DNA processing methods are improved. Since the 16s rRNA assay had sensitivity beyond culture and detected all fatal cases of melioidosis, the test may be useful for diagnosis of septicemic cases.

Single nucleotide polymorphism

A duplex TaqMan qPCR study with an assay accuracy of 100% was developed to detect and differentiate *B. pseudomallei* and *B. mallei* by targeting a SNP in a putative antibiotic resistance gene (P27) (U'Ren et al., 2005). The P27 method was later reported to identify a *B. oklahomensis* strain as *B. pseudomallei* and a *B. thailandensis*-like strain as *B. mallei* (Bowers et al., 2010). In response to the false positive results, Bowers et al developed a novel SNP-targeted duplex TaqMan qPCR test (BurkDiff) with a reported accuracy of 100%.

In a follow-up study, four qPCR procedures (Novak et al., 2006; Price et al., 2012), including BurkDiff, were evaluated for their ability to detect and differentiate *Burkholderia* using purified DNA (Price et al., 2012). BurkDiff was the most reliable test for *B. pseudomallei* detection, having a qPCR accuracy of 100%, but was also the most difficult test to interpret due to probe cross hybridization.

Additional targets

A 2-D gel electrophoresis analysis found various hypothetical proteins expressed solely by *B. pseudomallei* (Wongtrakoongate et al., 2007). A PCR test was developed targeting the gene of one of these hypothetical proteins (BPSL 1958), and reported an accuracy of 100%. This procedure needs further evaluation with *B. mallei* and additional *B. pseudomallei* and *B. thailandensis* strains, due to a limited sample size. In another study, a new assay targeting the BPSL 1958 hypothetical protein detected *Bp/Bm*, and had 100% accuracy for both pathogens (Koh et al., 2012). Therefore, this PCR assay may detect *Bp/Bm* rather than *B. pseudomallei*.

A PCR and TaqMan qPCR method was developed that targeted the aspartyl/asparaginyl β-hydroxylase gene (*lpxO*) for the specific detection of *B. pseudomallei* (Merritt et al., 2006). However, the *lpxO* TaqMan test was later described to detect *Bp/Bm* (Inglis et al., 2008). The *lpxO* PCR had a sensitivity and specificity of 88.9% (64/72) and 100%, respectively, and the *lpxO* qPCR had 100% accuracy.

Two follow-up studies evaluated Merritt et al's *lpxO* qPCR assay. One follow-up study adapted the qPCR for portable lab use and evaluated its ability to identify *B. pseudomallei* using purified DNA and soil sample suspensions (Inglis et al., 2008). The procedure was again reported to have 100% accuracy (A. Merritt, personal communication). In addition, the portable method detected *B. pseudomallei* in three of five direct soil suspensions from rice and rubber farms, but qPCR environmental accuracy cannot be determined because the soil samples were not culture-confirmed. Overall, the assay shows potential for portable detection of *B. pseudomallei*, but needs further evaluation for clinical and environmental use. The second follow-up study erroneously reported a *lpxO* qPCR sensitivity of 100% and a specificity of 87.8% (Kaestli et al., 2012). Kaestli et al believed the *lpxO* test to detect and differentiate *B. pseudomallei.* However, the *lpxO* qPCR actually detects *Bp/Bm*, therefore the assay sensitivity and specificity is 97.7% and 100%, respectively. This sensitivity is attributed to the assay's inability to detect 9/23 *B. mallei* strains. The same follow-up study was clinically evaluated on melioidosis patients, and had a test clinical sensitivity of 76% (30/50) and specificity of 100%. The same clinical samples were evaluated with six other assays, and the *lpxO* qPCR test was the third most clinically accurate for melioidosis detection. This procedure should not be used for *B. mallei* detection, but may be useful for *B. pseudomallei* detection on purified DNA and bacterial lysates. Although the *lpxO* qPCR method was the third most clinically accurate, this method should be supplemented with additional assays to confirm diagnosis.

Two TaqMan qPCR tests targeting TTS1 and transposase family protein (TFP) detected and differentiated *B. pseudomallei* and *B. mallei*, respectively, and had 100% qPCR accuracy (Zhang et al., 2012). The assays were also evaluated using clinical samples that were about 50 years old from patients with melioidosis or glanders. The tests had 100% patient accuracy. Not all of the clinical samples were culture confirmed, but overwhelming evidence suggests the samples were from melioidosis or glanders patients. These methods appear useful on purified DNA and clinical samples.

Three TaqMan qPCR procedures were developed for the differentiation of *B. pseudomallei, B. mallei*, and three

other CDC select agents (Rachwal et al., 2012). Two assays specifically detect *B. pseudomallei* and *B. mallei* while another detects *Bp/Bm*. The tests were evaluated in singleplex reactions and then adapted onto a novel TaqMan Array card. The *B. pseudomallei*-specific method detected all *B. pseudomallei* replicates and had a qPCR accuracy of 100%. However, the *B. mallei*-specific assay had a sensitivity of 89.3% (25/28 replicates) and a specificity of 100%. The *Bp/Bm* reaction had an accuracy of 100%. When the procedures were adapted for TaqMan array card use, the sensitivities decreased but the specificities remained at 100%. If these tests are used, it is recommended that all three methods in the singleplex format should be used on purified DNA instead of the TaqMan array card format. Additional bacterial strains, including *B. thailandensis,* should be evaluated by all three tests, and further evaluation of these assays for clinical and environmental use is needed.

For actual differentiation of *B. pseudomallei* and *B. mallei*, BurkDiff (Bowers et al., 2010) appears to be the best method for use with purified DNA. The test requires evaluation for clinical use. Purified DNA and clinical samples could also be used in Zhang et al's assay. Although the P27 SNP procedure (U'Ren et al., 2005) had false positives for *B. oklahomensis* and *B. thailandensis*-like strains, it could potentially be used in a clinical setting for melioidosis or glanders diagnosis.

For detection of *Bp/Bm,* the *fliC* and 16s rRNA methods (Tomaso et al., 2004; Tomaso et al., 2005) seemed to work well on purified DNA and crude bacterial lysates. The clinical use of the *fliC* procedure appeared useful, but the limited clinical sample size used mandates further evaluation with larger number of samples. Although the 16s rRNA assay patient sensitivity was low, the test had sensitivity beyond culture and detected all fatal cases of melioidosis. If sample processing methods are improved, the 16s rRNA assay may be a reliable method for diagnosis of septicemic cases. Kunakorn and Markham's 16-23s ITS non-nested assay appears useful on purified DNA, bacterial lysates, and clinical samples. However, further evaluation of the assay is needed due to the small clinical sample sizes used in the follow-up studies. An assay recommendation cannot be made for environmental samples because the existing studies, with the exception of one, were not evaluated for environmental use. The single environmental follow-up study of Merritt et al's *lpxO* assay did not compare test results with culture (Inglis et al., 2008), therefore the assay's potential environmental use is unknown.

B. pseudomallei and B. thailandensis

B. pseudomallei and *B. thailandensis* co-localize in the environment and produce similar results using routine diagnostic tests. Because they differ greatly in pathogenicity, the ability to differentiate these two species would be clinically useful.

Flagellar structural protein

A PCR assay, targeting a variable domain of *fliC*, was developed to differentiate *B. pseudomallei* and *B. thailandensis*, and had 100% accuracy (Wajanarogana et al., 1999). Differentiation of the species is determined by product size (191 bp or 179 bp), which is not easily distinguishable by gel electrophoresis. Therefore, Wajanarogana et al recommended an 8% acrylamide gel to increase size resolution.

Two studies used modified forward or reverse primers from Wajanarogana et al's method (Sonthayanon et al., 2002; Kao et al., 2003). Therefore, these studies could be considered novel assays, but due to the similarities of the primers they are best classified as follow-up studies. One follow-up study of Wajanarogana et al's method reported 100% accuracy (Sonthayanon et al., 2002). Two follow-up studies evaluated the method's ability to be used on environmental samples (Chen et al., 2002; Sonthayanon et al., 2002). The *fliC* assay reported 100% sensitivity on environmental samples for both studies, and also co-detected *B. pseudomallei* and *B. thailandensis* inoculated soil samples in one study (Sonthayanon et al., 2002). The follow-up studies provided from Sonthayanon et al and Chen et al had environmental specificities of 100% on inoculated soil samples and 0% (7/7) on collected soil samples.

Wajanarogana et al's *fliC* test has been shown to be useful for differentiation of *B. pseudomallei* and *B. thailandensis* on purified DNA and bacterial lysates, but the test needs further evaluation with *B. mallei* strains and clinical samples. The same environmental samples from Chen et al's follow-up study were evaluated with additional PCR methods (Dharakul et al., 1999; Winstanley and Hart, 2000), and false positives were found (Chen et al., 2002). Wajanarogana et al's *fliC* assay may have sensitivity beyond culture for soil samples, and therefore, the test may have higher environmental specificities than previously reported. Wajanarogana et al's procedure is also recommended for differentiation of *B. pseudomallei* and *B. thailandensis* in environmental samples. Chen et al recommends the *fliC* method with the possible addition of another PCR method (Dharakul et al., 1999) for detection of *B. pseudomallei* in soil samples. In fact, two epidemiological studies used Wajanarogana et al's and Dharakul et al's assays and reported that PCR was usually inferior to serology, but superior to culture in detecting *B. pseudomallei* in soil samples (Su et al., 2007; Chen et al., 2010). Chen et al's epidemiological study revealed 53 soil samples that were confirmed positive by Wajanarogana et al's and Dharakul et al's tests, but were negative by culture, indicating PCR sensitivity beyond culture (Chen et al., 2010). These results indicate Wajanrogana et al's test to be accurate, and likely a better indication than culture of the presence of *B. pseudomallei* in soil.

Single nucleotide polymorphisms

Two TaqMan duplex qPCR assays, 122018 and 266152, were developed to differentiate *B. pseudomallei* and a complex comprising *B. thailandensis, B. thailandensis*-like species, and *B. oklahomensis* (*Bt/Btl/Bo*), by targeting a SNP (Price et al., 2012). Overall, 122018 produced a sensitivity of 100% and a specificity of 98.4%, due to six false positives detected by the *B. pseudomallei* specific probe. An ambiguous detection of one *B. pseudomallei* strain was generated by 266152, and therefore provided a qPCR accuracy approaching 100%. In a follow-up study, 266152 and six other assays were evaluated for their abilities to detect *B. pseudomallei* using purified DNA and clinical samples (Kaestli et al., 2012). A test accuracy of 100% and a clinical sensitivity and specificity of 68% (34/50) and 100%, respectively, were produced by 266152. Consequently, 266152 and another test tie in rank for being

second to last in clinical accuracy. It appears evident that 266152 should not be used for melioidosis diagnosis. However, 266152 may be useful for the detection of *B. pseudomallei* in purified DNA, due to the assay's accuracy approaching 100%. The 122018 method had a higher sensitivity than 266152, and therefore, 122018 could help supplement the sensitivity of 266152.

Both the *fliC* and SNP procedures are reliable methods for differentiation of *B. pseudomallei* and *B. thailandensis* in purified DNA. The *fliC* assay's specificity has not been as heavily evaluated as that of the SNP tests on purified DNA, but the *fliC* test has sensitivity beyond culture for the detection of *B. pseudomallei* in soil samples.

B. pseudomallei, B. mallei, and B. thailandensis

The genotypic and phenotypic similarities of the *B. pseudomallei* complex have fueled the creation of several assays aimed at differentiating these species.

16s rRNA

A multiplex nested PCR method, targeting a 16s rRNA variable region, was developed to differentiate *Bp/Bm* and *B. thailandensis*. This assay had 100% accuracy (Dharakul et al., 1999). The procedure was also evaluated for clinical use on buffy coat samples, and reported 100% clinical and patient accuracy.

In a follow-up study, Dharakul et al's assay was used to further validate a suspected *B. thailandensis* strain (Winstanley and Hart, 2000). An additional follow-up study evaluated the test's ability to distinguish these organisms in environmental samples, and reported 100% environmental accuracy on inoculated soil samples (Chen et al., 2002). However, collected soil samples had an environmental PCR sensitivity and specificity of 100% and 0% (2/2), respectively.

Dharakul et al's procedure appears useful for differentiation of *Bp/Bm* and *B. thailandensis* using purified DNA and clinical buffy coat samples. In addition, the conclusion for environmental use of Wajanarogana et al's *fliC* procedure as previously described, can also be made about Dharakul et al's 16s rRNA assay, despite the different studies and data provided.

Repetitive element

A PCR study which targeted a repetitive element had 100% accuracy (Liu et al., 2002) in differentiating species within the *B. pseudomallei* complex. The PCR products ranged from 400-700 bp for *B. mallei* and *B. pseudomallei* strains, while *B. thailandensis* strains had a uniform 402 bp product. The product size overlaps make differentiation between the species difficult. However, differentiation of *Bp/Bm* and *B. thailandensis* may be possible, if a highly resolving gel is used.

Flagellin structural protein

A PCR-RFLP targeting *fliC* was developed for detecting *Bp/Bm* and *B. thailandensis,* and the method had 100% accuracy (Sprague et al., 2002). Use of restriction enzymes is not necessary to differentiate *Bp/Bm* from *B. thailandensis* if a highly resolving gel is used (H. Neubauer, personal communication). This test was used in another study to confirm *Bp/Bm* strains (Schmoock et al., 2009).

A nested PCR assay was developed that detects and differentiates the *B. pseudomallei* complex and *Burkholderia* genus by targeting *fliC* and *rpsU* 21, respectively (Hagen et al., 2002). Two of the four *fliC* primers used in this study were previously used by Sprague et al. The *fliC* procedure had 100% accuracy. In addition, the assay had a diagnostic sensitivity and specificity of 85.7% (6/7 spleens) and 100%, respectively, on inoculated mouse samples. Sequencing data from the false negative spleen isolate identified the organism as *B. plantarii.* Therefore, this PCR has 100% clinical accuracy. The bacteria from the spleen were uncultivable, indicating PCR sensitivity beyond culture. This method may be useful for *B. pseudomallei* detection on crude bacterial lysates and clinical samples, however only one strain from each species was tested, and therefore additional strains should be evaluated by this assay.

Tat domain, 70-kDa, and 12-kDa proteins

A multiplex PCR assay was developed to differentiate *B. pseudomallei, B. thailandensis,* and the *B. cepacia* complex by respectively targeting the genes of the Tat domain, 70-kDa, and 12-kDa proteins (Ho et al., 2011). The assay had 100% accuracy for *B. pseudomallei* or *B. thailandensis* using purified DNA. The procedure also had a clinical accuracy of 100% for a single *B. pseudomallei* and *B. thailandensis* inoculated sputum sample (P. Woo, personal communication). In addition, the assay was evaluated for its ability to be used on environmental samples, and the test was able to detect and co-detect *B. pseudomallei* and the *B. cepacia* complex with 100% accuracy based on sequencing results. The assay was not evaluated with *B. mallei* strains, but one study indicated *B. mallei* detection (Koh et al., 2012). Therefore, this method may actually differentiate *Bp/Bm, B. thailandensis*, and the *B. cepacia* complex. The test appears useful when used on purified DNA, clinical, and environmental samples. However the procedure needs further evaluation for *B. pseudomallei* detection in clinical use, due to the study's evaluation of only inoculated sputum samples.

From the assays described above, a recommendation cannot be made because 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.

Indirect differentiation assays

Indirect assays usually involve at least one primer set that is species-specific and additional primers to detect a complex of species. When the primers are combined, a unique amplification profile is created that indirectly identifies a species within the complex.

B. pseudomallei and B. mallei

23s rRNA

A PCR assay targeting a 23s rRNA SNP was developed with one procedure detecting *Bp/Bm* and the other detecting *B. mallei*. Both tests had 100% accuracy (Bauernfeind et al., 1998). In a follow-up study, the *B. mallei* assay was evaluated and confirmed to have 100% sensitivity. However, the follow-up study recommended the procedure be used only on purified DNA (Antonov et al., 2004). While these methods may be useful on purified DNA, additional bacterial

strains including *B. thailandensis* should be evaluated and the clinical potential of these assays should also be examined.

Tkachenko et al's two 23s rRNA-targeted tests, as previously described, were used as components of a novel nested PCR assay to increase detection sensitivity of *Bp*/*Bm* (Antonov et al., 2004). Nested and non-nested procedures were evaluated and only three tests reported 100% accuracy. Two of these assays are non-nested methods that detect *Bp/Bm*, B23 s5-a6 and B23 s7-a8, and the other assay is a nested procedure that detects *B. mallei.* One of the primer sets used in the nested *B. mallei* test has reported false positive detection for *B. pseudomallei* and *B. cepacia* strains, and therefore may not be useful for specific *B. mallei* detection. These non-nested tests also had 100% accuracy on inoculated environmental and clinical samples. A follow-up study of Antonov et al's B23 s5-a6 test reported an accuracy approaching 100% (Altukhova et al., 2007). Only the two non-nested procedures, B23 s5-a6 and B23 s7-a8, should be used for detection of Bp/Bm in purified DNA, environmental and clinical samples.

An assay by Bauernfeind et al appeared useful for indirect detection and differentiation of *B. pseudomallei* and *B. mallei* on purified DNA. Although a follow-up study did not recommend Bauernfeind et al's assays for clinical use, not enough data was reported to evaluate this claim, and therefore additional clinical follow-up studies may be useful. Tkachenko et al's assays are not recommended for detection and differentiation of *B. pseudomallei* and *B. mallei* because of the inaccuracies of the *B. mallei* assays. Consequently, the two non-nested procedures mentioned previously would be more useful for *Bp/Bm* detection in purified DNA, environmental, and clinical samples.

One follow-up study evaluated various procedures for their ability to detect *B. pseudomallei* and *B. mallei* in purified DNA, environmental and inoculated clinical samples (Antonov et al., 2008). All of the assays evaluated had 100% accuracy on purified DNA and inoculated clinical samples, with a detection limit of 10-10^2 genomic equivalents. PCR detected inoculated clinical samples beyond culture. Environmental samples were also tested, but accuracies were not reported. Although Antonov et al evaluated several PCR assays, very little detail was described on methodology and results. Therefore, this study will not be discussed further.

B. pseudomallei, B. mallei, and *B. thailandensis*

Type three secretion

Eight genes from type three secretion gene clusters 1, 2, and 3 (TTS1, TTS2, TTS3) were targeted for PCR development to differentiate the *B. pseudomallei* complex (Rainbow et al., 2002). Two of these gene targets experienced amplification difficulties (orf4/orf5 and orf1-bpscQ gap), and were modified for dot blot use (C. Winstanely, personal communication). Therefore, only six gene targets will be discussed further. Three of the six targets were used for *B. pseudomallei* identification, but only the *orf11* and *orf7* targeted tests had 100% accuracy. The remaining three targets were used in assays to detect *Bp/Bm* and the *B. pseudomallei* complex, and all three tests had 100% PCR accuracy. These assays need further evaluation using additional species including *B. mallei* strains.

Using gene targets from the TTS1 and TTS2 as previously described (Rainbow et al., 2002), three TaqMan qPCR procedures were developed to differentiate the *B. pseudomallei* complex (Thibault et al., 2004). The *orf11* test specifically detected *B. pseudomallei*, the *orf13* assay detected *Bp/Bm*, and the *bpscU2* method detected the *B. pseudomallei* complex. All assays had 100% accuracy.

Two follow-up studies evaluated Thibault et al's *orf11* test, and reported a sensitivity of 100% and a specificity approaching 100%. The four false positives detected were one *B. sordidicola,* one *B. mallei* (late C_T value), and two *B. thailandensis* strains (Tomaso et al., 2006; Kaestli et al., 2012). In two follow-up studies, the *orf11* method was evaluated for its ability to detect *B. pseudomallei* in clinical samples. Both follow-up studies reported low diagnostic sensitivities of 46.4% (13/28) (Supaprom et al., 2007) and 70% (35/50) (Kaestli et al., 2012). In addition, both follow-up studies had a diagnostic specificity approaching 100% (Supaprom et al., 2007; Kaestli et al., 2012). Only two false positive clinical sample isolates were detected by the *orf11* reaction, and the same false positives were also identified by the 8653 and 9438 assays, as previously described (Supaprom et al., 2007). Therefore the diagnostic specificity of the *orf11* test is likely to be 100%. In addition, two follow-up studies also evaluated Thibault et al's *orf13* assay. One follow-up study reported a sensitivity of 100% and a specificity of 97.6% (3/125), with the three false positives being identified as *B. caribensis, B. phenazinium,* and *B. thailandensis* strains (Tomaso et al., 2006). The second follow-up study evaluated the *orf13* method using clinical samples from three glanderous horses, and reported a patient sensitivity and specificity of 0% (0/1) and 100%, respectively. However, the same follow up study detected *B. mallei* in all three horses using their *fliP* assay and a CFT (Scholz et al., 2006). Therefore the patient sensitivity of the *orf13* test would still remain 0% (0/3) with an unknown specificity.

Thibault et al's *orf11* and *orf13* assays showed great sensitivity, but their specificities were less than 100%. If these tests are used, an additional *B. pseudomallei*-specific method should be added when evaluating samples of purified DNA or crude bacterial lysates. The *orf11* and *orf13* methods also had a low clinical sensitivity, but the *orf11* test had a clinical specificity of 100%. The *orf11* assay was evaluated with six other qPCR procedures for their abilities to be used on purified DNA and clinical samples, and the *orf11* procedure ranked fourth in clinical accuracy (Kaestli et al., 2012). Therefore the *orf11* and *orf13* tests are not recommended for clinical use.

Repetitive element and serine metalloprotease

A multiplex PCR method was developed to differentiate the *B. pseudomallei* complex using three primer sets (Lee et al., 2005). One primer set, targeting a 10 bp repetitive element (Liu et al., 2002), was used to detect the *B. pseudomallei* complex. A second primer set was used to detect *B. thailandensis* by targeting sequences within the *B. pseudomallei* complex amplicon. The last primer set, targeting *mprA,* was used to detect *B. pseudomallei/B. thailandensis*. The assay's accuracy was reported to be 100%, and direct PCR of overnight cultures was also possible. In a follow-up study, Lee et al's procedure was evaluated for its ability to distinguish these organisms in

clinical specimens taken from three glanderous horses. The test had a 0% clinical sensitivity (0/1) and 100% specificity (Scholz et al., 2006). In addition, the conclusions from the *fliP* assay and CFT, as previously described in a follow-up study of Thibault et al's *orf13* procedure, also apply to Lee et al's assay. This method may be useful for purified DNA and crude bacterial lysates only. Due to a limited sample size, its clinical usefulness remains unknown.

Flagellar structural protein

Two PCR tests targeting *fliC* were developed, with one assay detecting the *B. pseudomallei* complex and the other detecting *Bp/Bm* (Altukhova et al., 2007). If used together, *B. thailandensis* was indirectly differentiated from *Bp/Bm*, and both tests reported 100% accuracy. Only the *Bp/Bm* procedure was evaluated for its use on clinical specimens from inoculated hamsters. Clinical samples from the inoculated animals eliciting acute and sub-acute symptoms of pulmonary melioidosis and glanders were processed using PCR, and PCR proved to be more sensitive than culture. In addition, PCR predominantly detected pathogens in clinical specimens from acute and sub-acute disease states faster than culture. This study also showed that certain clinical samples were better suited for PCR detection. By day three, culture detected 100% of only infected lung specimens, whereas PCR detected these same infected lung specimens, as well as 40% of liver and spleen specimens on the same day. These assays were useful for the detection of both pathogens, using purified DNA. The *Bp/Bm* assay's clinical sensitivity was low, but PCR had better sensitivity than culture for both pathogens from inoculated hamster samples. Therefore, it is recommended that this *Bp/Bm* test be combined with additional assays that have proven clinically effective, for the detection of *B. pseudomallei* and *B. mallei* in clinical samples.

narK and *gltB*

A standard PCR and a six probe molecular beacon qPCR assay were developed to detect and differentiate *B. pseudomallei, B. mallei*, and *B. thailandensis/B. oklahomensis,* targeting the *narK* and *gltB* genes (Wattiau et al., 2007). The standard PCR procedure detects all four species in a complex, and the molecular beacon qPCR assay differentiates all four species. The standard PCR method targeting *narK* reported 100% accuracy for *B. pseudomallei* complex detection. The intention of the *gltB* target was to specifically detect the four *Burkholderia* species in a complex (P. Wattiau, personal communication), while the conventional PCR test detected all *Burkholderia* species except *B. phenazinium.* Therefore the *gltB* test had a sensitivity of 100% and specificity of 12.5% (14/16). From these results, it appears *gltB* may be a useful *Burkholderia* genus target. SNPs in the *gltB* and *nark* genes were targeted for a qPCR molecular beacon procedure. Indirect qPCR differentiation was possible between *B. pseudomallei*, *B. mallei,* and *B. thailandensis/B. oklahomensis*, and all molecular beacon probes had 100% accuracy. The qPCR assay specificity needs further evaluation relative to both sample type and sample size. In addition, *B. oklahomensis* was not tested in this study, but later evaluations indicated that both methods detected *B. oklahomensis* strains (P. Wattiau, personal communication). The six molecular beacon probes make *B. pseudomallei* complex detection costly, and therefore this assay is not recommended for *Burkholderia* detection in purified DNA samples. However the molecular beacon assay is able to detect three two-locus allelic profiles of *B. pseudomallei*. In addition, the molecular beacon assay results from boiled cell suspensions were comparable to those using purified DNA, and therefore this test may be useful clinically.

Additional targets

Six different genes were targeted in a microarray-based test to differentiate the *B. pseudomallei* complex and other *Burkholderia* species (Schmoock et al., 2009). Although the assay is designed for microarray detection, primers were developed for DNA amplification that could provide possible PCR targets. Only two of the six genes specifically detected a single species (*B. mallei*) and the remaining four primer sets detected a complex of *Burkholderia* species. All procedures had 100% sensitivity and five of the six assays had 100% specificity.

Four genes were used to develop a multiplex PCR method to detect and differentiate the *B. pseudomallei* and *B. cepacia* complex (Koh et al., 2012). The targets were designed to specifically detect *B. pseudomallei*, *Bp/Bm,* the *B. pseudomallei* complex, and the *B. cepacia* complex. The three tests associated with detection of the species from the *B. pseudomallei* complex had 100% PCR accuracy.

Bauernfeind et al's 23s rRNA-targeted assay appears useful for the indirect differentiation of *B. pseudomallei* and *B. mallei,* but requires clinical evaluation. Liu et al's, Schmoock et al's, and Koh et al's assays allow for indirect differentiation of all three species of the *B. pseudomallei* complex, and appear useful for detection of these species in purified DNA. These three tests require clinical evaluation, and Schmoock et al's assay requires additional *B. pseudomallei* complex evaluation using purified DNA, because of a limited sample size.

Although Altukhova et al's *fliC* assay has sensitivity beyond culture for *B. pseudomallei*- and *B. mallei*-inoculated hamster samples, it was unable to differentiate all three species of the *B. pseudomallei* complex. This test is the only indirect differentiation assay that has detection capability for the agents of melioidosis and glanders. Wattiau et al's molecular beacon method may be useful for *B. pseudomallei* detection in clinical samples, because of its ability to detect three variants of *B. pseudomallei*, but the assay requires clinical evaluation.

Conclusions

Glanders and melioidosis are serious diseases with high mortality rates. The low infectious dose and bioweapon potential of *B. mallei* and *B. pseudomallei*, necessitates the need for rapid and accurate detection methods. Developing assays that reduce the diagnostic time, could decrease morbidity and mortality rates in melioidosis- and glanders-endemic areas.

Although PCR-based procedures have revolutionized microbial detection due to their accuracy, sensitivity, and speed, they do have limitations. The high sensitivity of PCR-based assays can also be a drawback. False positives can arise from background contamination from external sources of DNA, such as the "carry-over" products from earlier PCR runs (Fredricks and Relman 1999, Yang and

Rothman 2004). Conversely, false negatives can occur due to inadequate removal of PCR/qPCR inhibitors. PCR-based assays are usually designed around a well-conserved gene. It is possible, especially in newly emerging pathogens, for mutations to occur in the gene of interest, which can then compromise the test (Klein 2002). Restricting bacterial detection to a single target is another limitation of PCR-based tests. These issues can be overcome by developing multiplex procedures. Although PCR-based assays have the potential to provide high-throughput, the limitations associated with possible gene mutations, false positives, false negatives, sample processing, and the need to validate with other established assays, can decrease the overall throughput of the entire PCR/qPCR process.

False positives have been discussed in this review. However, some of these assays have reported sensitivity beyond culture for clinical and environmental samples that correlates with serological, radiological, and/or additional PCR-based assays. Detection of non-viable *B. pseudomallei* in clinical samples from confirmed melioidosis patients has also been observed. Therefore, a positive result from some of the test methods discussed, may be a better indication of the presence of *Burkholderia* species in clinical and environmental samples, than culture. In addition, one study observed 45% of septicemic melioidosis patients exhibited less than 1 CFU/mL of *B. pseudomallei* in their blood samples (Walsh et al., 1995), which reinforces the need for sensitive testing methods such as PCR-based assays. Conversely, false negatives have also been mentioned in this review. Instances of 100% clinical/environmental accuracy have been reported by the tests previously described, but high repeatability of these accuracies has not been observed in all follow-up studies. Sample processing methods, sample types, and the collection of samples during antibiotic treatment have likely caused the variability in assay accuracies. The PCR-based method is not the primary issue for false negatives, rather, lack of optimization and normalization of sample processing methods is likely the reason. As a result of false positives and negatives, PCR-based tests are often validated by other established PCR/qPCR assays, which decrease throughput.

The increasing affordability of sequencing technologies has created databases containing annotated genomes of *Burkholderia* species. These *Burkholderia* sequences may identify variability or mutations within a conserved gene, which will likely aid in developing better *B. pseudomallei* complex assays, by facilitating the development of degenerate primers. Of the 72 publications discussed, mutations in the form of gene deletions have affected assay sensitivity in only two studies. Ulrich et al's *bimA* PCR assay was unable to detect two avirulent *B. mallei* strains. Novak et al's TTS1 test and its follow-up studies detected a total of 2,572 *B. pseudomallei* strains, and had one false negative because of a reduced genome lacking some virulence loci. The *bimA* procedure's inability to detect avirulent *B. mallei* is not as vital as detecting virulent *B. mallei* strains. From these limited examples, it appears mutations within PCR-targeted genes have not significantly compromised the reliability of PCR-based tests for *B. pseudomallei* complex detection. *B. mallei* has evolved as a strict pathogen from *B. pseudomallei*. Despite the major evolutionary change of these two species, they continue to be identified as a complex in several PCR/qPCR methods. Therefore, gene mutations within the already genetically similar *B. pseudomallei* complex, may not drastically compromise the validity of PCR-based assays.

qPCR tests are able to resolve some of the limitations previously described because of the versatility of the internal probe. This method prevents the possible contamination of other reactions by eliminating the need for any post-PCR product manipulation. The probe provides additional specificity compared to methods that use only primers. The probe technology also allows for simultaneous detection of multiple targets which overcomes the problem of potential gene mutations at a single locus. However, such assays are difficult to implement due to the high degree of optimization that is required. Overall, qPCR assays are generally considered to have a large dynamic range, low interassay variation, and high reliability (Purcell et al., 2011).

PCR-based assay development depends heavily on time, trained personnel, funding, and equipment. Melioidosis endemic regions, especially rice-paddy communities and indigenous tribes, may not have access to these resources. However, competition has reduced initial start-up and operating costs of PCR/qPCR systems (Purcell et al., 2011). 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 areas.

The loop-mediated isothermal amplification (LAMP) technique is a potential cost-effective alternative to PCR-based tests. The LAMP method shares similar characteristics to those of qPCR. Without the need of thermocyclers, LAMP uses several primers to amplify specific regions on the gene target and utilizes fluorescent dyes for visual interpretation by the naked eye. One study reported the development of a LAMP assay for *B. pseudomallei* detection, and showed it to be more reliable for clinical use when compared to Novak et al's robust *orf2* TTS1 qPCR procedure (Chantratita et al., 2008). Therefore, the LAMP method has potential to become an effective diagnostic tool for *B. pseudomallei* complex detection.

The future of diagnostic testing is constantly shifting towards a molecular approach. Although several PCR-based studies have reported possible sensitivities beyond culture for the members of the *B. pseudomallei* complex, PCR-based methodologies alone, with current established procedures, cannot be used with 100% confidence. Therefore, *B. pseudomallei* complex PCR-based assays in their current state, should be complimented with culture and/or additional tests, until more research proves otherwise.

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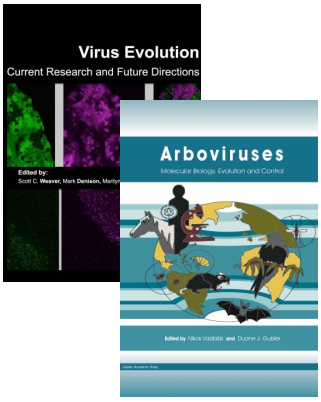
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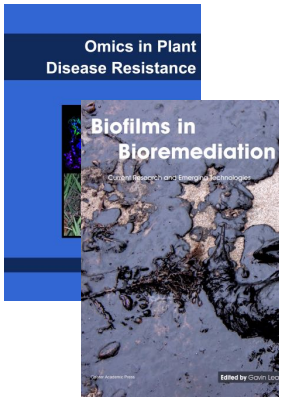
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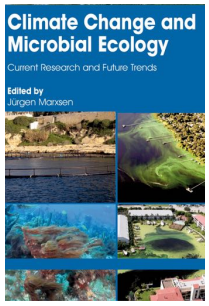
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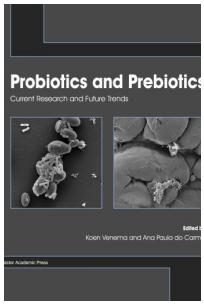
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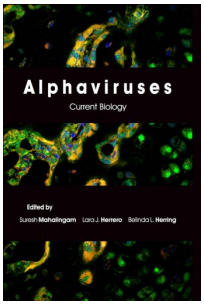
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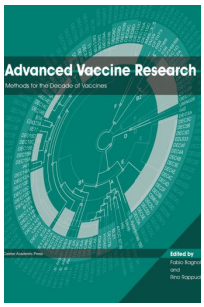


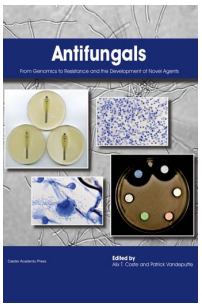












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