TECHNICAL REPORT

Strategies for PCR based detection of *Burkholderia pseudomallei* DNA in paraffin wax embedded tissues

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Recently, several cases of melioidosis imported to Europe have been reported. The diagnosis of the acute or chronic infection remains challenging. This report describes an optimised protocol for fast and reliable DNA preparation for use in two different polymerase chain reaction (PCR) assays, namely: (1) a seminested PCR assay targeting a genus specific sequence of the ribosomal protein subunit 21 (rpsU) gene and (2) a nested PCR assay targeting the gene encoding the filament forming flagellin (fliC). Various strains of *Burkholderia* spp, strains of closely related genera, and spleen tissue samples of experimentally infected mice were investigated. The combination of PCR and sequencing of the amplicons resulted in high sensitivity and specificity. These procedures may allow rapid, sensitive, and reliable detection of *B pseudomallei* DNA in routinely formalin fixed and paraffin wax embedded samples, thus providing a safe diagnostic tool and avoiding the cultivation of a risk group 3 agent. In addition, this method could be useful for retrospective histopathological investigations.

Melioidosis, a potentially fatal infection in humans and animals, is caused by *Burkholderia pseudomallei*, a motile, non-spore forming, Gram negative bacterium.[1] This saprophyte is found in surface water and the soil of endemic areas in tropical and subtropical regions between latitudes 20° north and 20° south. Melioidosis should also be suspected in travellers or refugees from endemic areas presenting with an acute febrile disease or symptoms of a chronic ulcerative or abscess forming infection.[2 3] Because of the rapidly growing number of newly described and phylogenetically related species, it is difficult to identify *B pseudomallei.* Consequently, melioidosis causes diagnostic problems in endemic and even more in non-endemic areas when imported.[3–6]

During the past decade, many efforts have been made to develop new molecular procedures to identify *B pseudomallei* from different specimens. Most polymerase chain reaction (PCR) approaches were sensitive, detecting as few as one bacterium/ml, but showed a lack of specificity. In subacute and chronic forms, when blood culture and PCR from blood samples are often negative, biopsy may be required, but histological findings are not specific.[2] The detection of *B pseudomallei* from tissue by means of PCR has not been described so far.

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The quality of the template DNA depends on the sampling and purification procedures. Conventional protocols for dewaxing, digestion, and purification of DNA from paraffin wax embedded tissues are time consuming and may cause considerable loss of DNA.[7] Therefore, we developed strategies for rapid and reliable DNA preparation from tissue samples, conserving the bacterial DNA, and thus allowing the amplification of specific sequences of either (1) the ribosomal protein subunit 21 (rpsU) gene or (2) the gene encoding the filament forming flagellin (fliC).

MATERIAL AND METHODS

BACTERIA

Bacterial strains (table 1) were cultured on nutrition agar, swabbed from the plates with sterile 0.9% NaCl solution, adjusted to 10^8 to 10^9 bacteria/ml, and heat inactivated.

Experimental infection and organ samples

A mouse model for experimental melioidosis has been established and described recently.[8] Spleen samples of SWISS mice infected with strain *B pseudomallei* 6068VIR were collected, and routinely formalin fixed and paraffin wax embedded at days 2, 3, 4, 5, and 7 after infection, from surviving animals only. A non-infected mouse served as the negative control. The bacterial load of the organs was determined by plating out on plate count agar (Merck, Darmstadt, Germany) and expressed as colony forming units (CFU)/g of organ. To determine the in vitro detection limit of the PCR, a bacterial suspension of *B pseudomallei* 6068VIR containing 5×10^8 CFU/ml was serially diluted one in five and inactivated, as described previously.[8]

DNA preparation

The bacterial suspensions were prepared according to the following protocol. For bacterial lysis, buffer contained 0.5% Tween™ 20 (ICI, American Limited, Merck, Hohenbrunn, Germany), 2 mg/ml proteinase K (Roche, Mannheim, Germany), 3.5mM $MgCl_2$, 15mM ammonium sulfate, and 60mM Tris/HCl (pH 8.5). Aliquots (10 µl) of the heat inactivated bacterial suspensions were resuspended in 200 µl of lysis buffer, incubated at 56°C for two hours to obtain a clear lysate, and boiled for 10 minutes.

Sections (5 µm thick) were cut from paraffin wax blocks to give 1 mg of tissue and dewaxed by xylene extraction. The samples were incubated for 10 minutes in xylene at room temperature (1200 µl, 2×) and 100% ethanol (1200 µl, 3×) while gently agitating the tube. After subsequent centrifugation at 13 000 ×*g* for 10 minutes the supernatant was removed and the samples were then air dried. For digestion of the tissues, 200 µl of lysis buffer was added. The samples were then incubated for one hour at 56°C and mixed continuously. A clear lysate was obtained and the reaction was stopped by boiling the sample for 10 minutes. No further DNA extraction was performed.

Abbreviations: CFU, colony forming units; PCR, polymerase chain reaction

Table 1 Results of the polymerase chain reaction (PCR) assays based on the rpsU gene and the fliC gene

Species	Source	rpsU PCR	fliC PCR
Burkholderia pseudomallei	ATCC 15682	179 bp	302 bp
B pseudomallei	ATCC 23343	179 bp	302 bp
B pseudomallei	6068VIR	179 bp	302 bp
B thailandensis	ATCC 700388	179 bp	302 bp
B. mallei	ATCC 23344	179 bp	302 bp
B cepacia	ATCC 25416^T	179 bp	–
B vietnamiensis	LMG 10929	179 bp	–
B gladioli	ATCC 10248	179 bp	–
B plantarii	LMG 9035	179 bp	–
B vandii	ATCC 51545	179 bp	–
Pseudomonas aeruginosa	DSM 1253	–	–
Ps fluorescens	IMB P162 human isolate	–	–
Ps stutzeri	NCTC 10450	–	–
Escherichia coli	DSM 301	–	–
Francisella tularensis	ATCC 29684	–	–
Stenotrophomonas maltophilia	IMB P26 human isolate	–	–
Staphylococcus aureus	DSM 346	–	–
Staphylococcus epidermidis	DSM 1798	–	–
	Spleen mouse 1 (2.09×10^6)*	179 bp	302 bp
	Spleen mouse 2 (2.21×10^7)*	179 bp	302 bp
	Spleen mouse 3 (69)*	179 bp	–
	Spleen mouse 4 (1.46×10^8)*	179 bp	302 bp
	Spleen mouse 5 (1.57×10^9)*	179 bp	302 bp
	Spleen mouse 6 (2.64×10^8)*	179 bp	302 bp
	Spleen mouse 7 (8.18×10^8)*	179 bp	302 bp
	Spleen control mouse	179 bp	–

ATCC, American Type Culture Collection, Atlanta, USA; DSMZ, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; IMB, Type collection Institut für Mikrobiologie, Munich, Germany; LMG, Laboratorium Microbiologie Gent Culture Collection, Belgium.
*Colony forming units/g of organ.

DNA amplification procedures

A 1 µl aliquot of the cleared bacterial lysate or of the tissue sample lysate was used as template for both PCR assays. PCR primers (table 2) were designed based on the previously published sequence of the rpsU and fliC genes of *B pseudomallei* strain E503 (NCBI accession number, AF030239). The PCR mixture contained 0.5 U Taq polymerase (Perkin Elmer, Weiterstadt, Germany), 1µM of primers, 1mM dNTP, 2.5mM $MgCl_2$ (rpsU PCR) or 1.75 mM (fliC PCR), 7.5mM ammonium sulfate, and 30mM Tris/HCl (pH 8.5). The rpsU PCR using the fup-1/fup-2 primers was performed under the following conditions: an initial denaturation step of 10 minutes at 94°C was followed by 35 cycles of denaturation for 60 seconds at 94°C, annealing for 60 seconds at 59°C, and elongation at 72°C for 60 seconds. An additional elongation step was performed for 10 minutes at 72°C. Further amplification of 0.5 µl aliquots was performed by seminested PCR using the rpsU-L2/fup-2 primers and applying the same conditions as the initial amplification except that annealing was carried out at 53°C. An already developed *B mallei*/*B pseudomallei*/*B thailandensis* specific filament forming flagellin (fliC) PCR assay based on primers fliC-1/fliC-2[9] was connected with a nested (inner) PCR assay using primers fliC-3/fliC-4. The fliC PCR was carried out as described previously.[9]

In every PCR a negative control (without DNA) and positive controls (DNA preparation of *B pseudomallei* 6068VIR and *B pseudomallei* ATCC 15682) were included. An 8 µl aliquot of the PCR mix was analysed by agarose gel electrophoresis and ethidium bromide staining.

Sequencing

Direct sequencing of PCR products was done on ABI 377 PRISM™ dye sequencing apparatus using the ABI PRISM dye terminator cycle sequencing ready reaction kit™, according to the manufacturer's instructions (Perkin Elmer Applied Biosystems, Weiterstadt, Germany). Alignment studies were done using CLUSTAL 5.0.

RESULTS

The bacterial load of the spleens expressed as CFU/g of organ ranged from 69 to 1.57×10^9 bacteria/g (table 1). No cultivatable bacteria were found in the spleen from the control mouse. The seminested rpsU PCR assay allowed the detection of one bacterium/ml using diluted bacterial suspensions and gave positive results with amplicons of 179 bp length for all (burkholderia) reference strains, but not for the other bacterial species tested (table 2). Positive results were obtained from the tissues of all the mice. The PCR products were analysed by direct sequencing. Only the DNA sequences of three *B pseudomallei* reference strains and one *B mallei* reference strain were available from NCBI GenBank. The sequencing results of the partly sequenced rpsU gene from seven other (burkholderia) reference strains were submitted to NCBI GenBank with the following accession numbers: AF447444,

Table 2 Polymerase chain reaction primers used for the amplification of bacterial DNA

Primer	Sequence
fup-1	5′-GTG GAG CTT CTT CGG CAG CAT-3′
fup-2	5′-ATG ACG ACG ATT CTT TTG AA-3′
rpsU-L2	5′-AGG CGC TTG TGC AGG CGC-3′
fliC-1	5′-GAT CGG CGG CGG CAT GGT TCA GA-3′
fliC-2	5′-CCG AGC GTT GCC TGC AGA TTG TT-3′
fliC-3	5′-GCA GCA CCG GCC AGG AGA CGA C-3′
fliC-4	5′-GCC CGT CTG CGT GCT GAT GTC-3′

Take home messages

- This report describes an optimised protocol for fast and reliable DNA preparation for use in two polymerase chain reaction (PCR) assays targeting a genus specific sequence of the ribosomal protein subunit 21 (rpsU) gene and the gene encoding the filament forming flagellin (fliC)
- The combination of two PCRs and sequencing of the amplicons resulted in high sensitivity and specificity
- These procedures may allow rapid, sensitive, and reliable detection of *B pseudomallei* DNA in routinely formalin fixed and paraffin wax embedded samples, thus providing a safe diagnostic tool and avoiding the cultivation of a risk group 3 agent

AF447445, AF447446, AF447447, AF447448, AF447449, and AF447450. The PCR products from mice numbers 1, 2, 4, 5, 6, and 7 showed the same sequence as strain *B pseudomallei* 6068VIR. In contrast, the sequence of the PCR product from mouse number 3 was homologous to the sequence of reference strain *B plantarii* LMG 9035, the PCR product obtained from the control mouse showing the same sequence as the reference strain *B vandii* ATCC 51545. Therefore, these PCR products were identified as contaminants.

The nested fliC PCR assay gave rise to amplicons of 302 bp in length for the *B pseudomallei*, *B thailandensis*, and *B mallei* reference strains, but not for members of other (burkholderia) species or the other genera (table 1). The nested fliC PCR gave positive results for the spleens from mice numbers 1, 2, 4, 5, 6, and 7, but not for the spleens from mouse number 3 and the control mouse. The identity of the amplicons was confirmed by direct sequencing of the PCR products. The sequences of all PCR products obtained originated from *B pseudomallei* DNA.

DISCUSSION

Here, we describe the development of a seminested PCR assay based on the sequences of the ribosomal protein subunit 21 (rpsU) gene[10] and of a nested PCR assay targeting the gene encoding the filament forming flagellin (fliC) for the identification of *B pseudomallei.* We showed that both genes are highly conserved in the species *B pseudomallei*. Both PCR assays have been used successfully for the detection of bacterial DNA in formalin fixed, paraffin wax embedded spleen tissue from infected mice. The preparation of the specimens with a simple, rapid, and cheap method (lysis using proteinase K) gave reliable results.

> "The high sensitivity and specificity recommend the use of this strategy for the diagnosis of melioidosis for retrospective histopathological studies"

We conclude that the combination of the two PCR assays with subsequent sequencing of the PCR products leads to a safe species specific identification of *B pseudomallei*, thereby avoiding the cultivation of this risk group 3 agent. The high sensitivity and specificity recommend the use of this strategy for the diagnosis of melioidosis for retrospective histopathological studies.

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