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Development of a Polymerase Chain Reaction Assay for Detection of *Burkholderia mallei*, a Potent Biological Warfare Agent

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ABSTRACT

Burkholderia mallei is the etiological agent of glanders, primarily a disease of equines. *B. mallei* is closely related to *B. pseudomallei*, the causative agent of melioidosis. Therefore, detection of *B. mallei* and its differentiation from *B. pseudomallei*, has always been troublesome. In present investigation, a *B. mallei* specific DNA sequence was identified by performing BLASTn search using ~ 3000 ORFs of *B. mallei* NCTC 10229. A polymerase chain reaction (PCR) assay with internal amplification control (IAC) was developed for detection of *B. mallei* and its differentiation from *B. pseudomallei*. The PCR assay could amplify a specific 224-bp fragment from all the six *B. mallei* strains used in the study, whereas other closely related organisms were tested negative. The detection limit of the assay was found to be 10 pg of purified DNA of *B. mallei.* Incorporation of IAC in the assay makes the results reliable as false negative results which may arise due to presence of PCR inhibitors, can be avoided. For validation, the assay was tested on tap water, Bengal gram and grass artificially spiked with *B. mallei.* The developed assay can be used as a simple and rapid tool for detection of *B. mallei*.

Keywords: *Burkholderia mallei, Burkholderia pseudomallei, glanders*, internal amplification control, polymerase chain reaction

1. INTRODUCTION

Burkholderia mallei is a gram-negative, obligate mammalian pathogen which causes glanders primarily in solipeds, including horses, donkeys and mules. Horses are considered to be the natural reservoir of *B. mallei*[1]. Camels, goats, rabbits, bears, cats, wolves, and dogs have been proven susceptible to glanders. Human glanders is scarcely reported and is associated with animal handlers, veterinarians and laboratory workers[2-4]. Humans get infected by contact with sick equids, contaminated fomites, tissues or bacterial cultures and the transmission is often through small wounds and skin abrasions. Inhalation of contaminated aerosols also results in infection. Laboratory-acquired infections can also occur during mishandling of cultures or samples[4]. The outcome of untreated infections leading to septicemia is uniformly fatal[5,6]. Glanders has been eradicated from most of the Western hemisphere countries; however, it is still endemic in Central and South America, Asia and the Middle East[1]. Recent glanders outbreaks have been reported from Turkey, United Arab Emirates, Iraq, Iran, Pakistan, China, Brazil, Bahrain, and India, thereby reflecting the actual area of endemicity[7]. *B. mallei* is resistant to many antibiotics. Hence, early detection of the agent is critical to ensure the administration of appropriate antibiotics[8]. *B. mallei* organisms are highly infectious as aerosol, and infection requires few organisms, offering the potential for intentional release as biological threat agent. The bacterium has been classified as category B bioterrorism agent by the Centers for Disease Control and Prevention, Atlanta.

Because of its potential for weaponisation, rapid and definitive detection of *B. mallei* is critical to ensure the administration of appropriate antibiotic therapy. Identification of causative agent employing classical microbiological and biochemical methods usually consume more than a week[9,10]. Accuracy of commercially available kits (API 20NE and RapID NF) for the identification of *B. mallei* has been reported to be 0–60 per cent[11], and they may give false positive results. *B. mallei* being deletion clone of *B. pseudomallei* shares ~ 99.5 per cent DNA-DNA sequence identity with the later[12]. Use of molecular and biochemical approaches for identification of *B. mallei* and its differentiation from *B. pseudomallei* has been troublesome because of high level of genetic, biochemical, and phenotypic similarities between the two species[12-15]. *B. mallei*-specific molecular assays did not exist for discriminating this pathogen from *B. pseudomallei* till year 2006. PCR based assays targeting *fliP* and $bimA_{Bm}$ genes were reported for definitive identification and differentiation of *B. mallei* from *B. pseudomallei* and other bacteria[16-19]. Later, $bimA_{Bm}$ gene orthologue was also reported in few strains of *B. pseudomallei* viz., MSHR668, MSHR172, MSHR491, MSHR33[20]. BurkDiff, a RT-PCR assay targeting a unique conserved region in *B. mallei* and *B. pseudomallei* genomes containing a single nucleotide polymorphism (SNP) differentiated the two species[21]. Previously, a quadruplex qPCR for detection and differentiation of *B. mallei* and *B. pseudomallei* has been developed[22]. Recently, a duplex PCR assay with fluorescent

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labelled primers was developed for clear distinction between *B. mallei* and *B. pseudomallei*/*B. thailandensis*[23]. The real time PCR based assays require sophisticated instruments and costly reagents. Development of simple and cost-effective PCR assays can be helpful in detection of pathogens in laboratories with resource-limited settings.

PCR is a simple, sensitive and rapid assay for detection of pathogens, but possibility of false-negative or false positive results are the most important limitations of this method. False-positive results in PCR assays can be avoided by using anti-contamination procedures. False-negative results due to PCR failures deny appropriate treatment and containment/ quarantine, which may lead to spread of infectious disease. Hence, a detection method should ensure that the negative results are truly negative. Inclusion of an internal amplification control (IAC) in the same sample reaction and its co-amplification with the target sequence would generate a control signal in absence of PCR failures. The general guidelines for PCR testing require inclusion of IAC in PCR reaction mixture for indication of PCR failures[24,25]. In present investigation, we describe a PCR based assay with IAC for detection of *B. mallei* from various artificially spiked samples.

2. MATERIALS AND METHODS

The work was carried out in high containment facility, a biosafety level 3 laboratory at Defence Research and Development Establishment, Gwalior, India.

2.1 Bacterial Strains and Growth Conditions

Various bacterial strains used in this study are listed in Table 1. Standard strains of *B. mallei* and *B. pseudomallei* were obtained from Central Public Health Laboratory, Colindale, London, U.K. Clinical isolates of *B. cenocepacia* were kindly provided by Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh. Standard cultures of *Pseudomonas putida* ATCC 49128, *P. aeruginosa* ATCC 9027 and *P. fluorescens* ATCC 13525 were procured from Hi-Media, Mumbai. Other bacterial strains used in the study were taken from DRDE, culture collection. All the bacterial cultures were grown at 37 °C in their respective media, procured from Difco, U.S.A and Hi-Media, India. Genomic DNA from the bacterial strains was isolated either by boiling method[26] or by DNA extraction kit (Qiagen, Hilden, Germany).

2.2 Identification of *B. mallei* Specific DNA Sequence

About 3000 ORFs of *B. mallei* NTCC 10229 strain were retrieved from Pathema-*Burkholderia* bioinformatics resource centre (http://pathema.jcvi.org/cgi-bin/Burkholderia/PathemaHomePage.cgi) and BLAST search was performed using online BLASTn search offered by the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/) for identification of *B. mallei* specific DNA sequence(s).

On the basis of BLASTn search, a sub-region of ORF *BMA10229_0375* of *B. mallei* NCTC 10229 was selected as *B. mallei* specific DNA sequence for PCR based detection. *BMA10229_0375* ORF is present on Plus (+) strand of chromosome II of *B. mallei* NCTC 10229 between coordinates 375855 and 376301. This 447-bp ORF is present in *B. mallei* strains and absent in closely related *B. pseudomallei* strains except *B. pseudomallei* K 96243, where in initial 217 bases have 95 per cent sequence identity with sequence of *BMA10229_0375* (from coordinates 375857 to 376071). The ORF, however is disrupted by IS element insertion in *B. pseudomallei* K 96243[14]. A 244 bp DNA sequence between coordinates 376072 and 376315 was selected as *B. mallei* specific DNA sequence and Bms primers were used to amplify a sequence of 224-bp (coordinates 376092 to 376315).

Table 1. List of primers and their nucleotide sequence

Primers	Primer name	Nucleotide sequence (5′→ 3′)
Bms primers	Bms FP	GTGATGGACCGCTGTATCG
	Bms RP	ATTCACTGCAAGCGTCAGG
IAC primers	IAC FP	GTGATGGACCGCTGTATCGGT GCCACCTAAATTGTAAGCG
	IAC RP	ATTCACTGCAAGCGTCAGGTGA CCGCTACACTTGCCAGC

2.3 Primer Design and IAC

On the basis of BLAST search, a subregion of ORF *BMA10229_0375* of *B. mallei* NCTC 10229 was identified as *B. mallei* specific DNA sequence. Two sets of primers, Bms and IAC were designed for specific amplification of *B. mallei* (224 bp) and pBluescript SK (+) phagemid (449 bp), respectively (Table 1). The IAC primers had 5′ overhanging ends, which were identical to Bms primers used to amplify *B. mallei* specific DNA sequence, whereas the 3′ ends were complementary to a chosen DNA sequence of pBluescript SK (+) phagemid.

The PCR reaction mixture for generation of IAC DNA contained 1 × PCR master mix (MBI Fermentas, Vilnius, Lithuania), 1 μM of each of the primers, and 500 pg of pBluescript SK (+) phagemid template DNA. The reaction procedure consisted of 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 60 °C for 30 s and extension at 72 °C for 30 s. The DNA was denatured for 4 min in the beginning and finally extended for 5 min at 72 °C. PCR product was purified using commercially available kit (Fermentas). The concentration of IAC DNA was determined spectrophotometrically at 260 nm and was stored in Milli-Q water at -20 °C. The following equation was used to calculate the copy number of the PCR product.

Number of copies of PCR fragment per microlitre = weight of PCR fragment (in g μl^{-1}) × (6.023×10^{23})/(660 g mol^{-1} × number of base pairs of PCR fragment).

2.4 PCR Amplification of *B. mallei* Specific Sequence

PCR was carried out in 25 μl reaction containing 1× PCR master mix, 1 μmol l^{-1} of Bms FP and Bms RP primers, 1000 copies of IAC DNA, and 100 pg of template DNA of *B. mallei* NCTC 10230. Various concentrations of IAC DNA were tried before choosing 1000 copies per reaction. PCR was taken through 38 cycles in Bio-Rad iCycler (Thermal cycler) (Bio-Rad Laboratories, Hercules, CA, USA) at 94 °C for 1 min (denaturation), 59 °C for 30 s (annealling) and 72 °C for 30 s

(extension). Gradient temperature from 50 °C to 61 °C was initially used before finalising 59 °C as annealing temperature. The DNA was denatured for 4 min in the beginning and finally extended for 5 min at 72 °C. PCR products were analysed in 1.5 per cent agarose.

2.5 Determination of Detection Limit, Specificity and Robustness of PCR Assay

To determine the detection limit of PCR assay, dilutions of genomic DNA of *B. mallei* were prepared. The PCR reaction was processed with known amount of genomic DNA (10 ng/reaction to 0.1 pg/reaction through 1 ng, 100 pg, 10 pg, 1 pg/reaction) in separate PCR tubes along with 1000 copies of IAC, according to the conditions presented earlier.

The specificity of Bms primers was checked against bacterial cultures shown in Table 1, by incorporating 10 ng of template DNA of respective bacterium along with all other PCR ingredients. Procedure for PCR was essentially same as described in the previous section.

Robustness of the PCR assay was determined by using suboptimal concentrations of the reagents and IAC template (20 per cent lower or 20 per cent higher). The experiments were performed at annealing temperatures of 57 °C, 59 °C, and 61 °C. All other conditions were kept constant as described earlier.

2.6 Validation of PCR Assay with Environmental Samples Artificially Inoculated with *B. mallei*

Duplicate samples of tap water and grass were collected from our laboratory in sterile containers. Bengal gram was collected from local market. Serially 10-fold diluted overnight grown *B. mallei* culture (1 ml) was spiked in 9.0 ml of water, 2.0 g of Bengal gram (soaked overnight in Milli-Q water), or 2.0 g of grass in such a way that different aliquot of each sample received *B. mallei* bacteria from 10^1 to 10^6 through 10^2, 10^3, 10^4, 10^5 CFU/ml or g. After keeping at room temperature for 20 min, 1 volume of double strength glycerol dextrose broth and penicillin (200 Units/ml) was added to each sample and incubated at 37 °C in an incubator shaker for 24 h. One milliliter sample was collected from spiked material after 8 and 24 h of growth and DNA was extracted in 50 µl of sterile Milli-Q water by boiling method, of which 2 µl was used as template DNA for detection by PCR assay. The exact number of spiked bacteria was determined by plate count method.

3. RESULTS

3.1 PCR Assay Optimisation

IAC DNA (449-bp) was prepared by PCR and its copy number per microlitre was determined. Temperature gradient (50 °C to 61 °C) PCR was performed to optimise the annealing temperature of Bms primers. The annealing temperature of 59 °C was finally selected. One thousand copies of IAC were found to be optimum for co-amplification in PCR assay with 10 pg of purified *B. mallei* NCTC 10230 genomic DNA.

3.2 Analytical Sensitivity, Specificity and Robustness of PCR Assay

For determination of analytical sensitivity of PCR, ten-fold serially diluted genomic DNA (10 ng/µl to 0.1 pg/µl) of *B. mallei* was prepared and 1 µl of each of dilutions was added to separate PCR tubes along with 1000 copies of IAC. The PCR assay could detect 10 pg of *B. mallei* genomic DNA as indicated by presence of 224-bp band on agarose gel electrophoresis of PCR products (Fig. 1 Different amounts of purified genomic DNA of *B. mallei* NCTC 10230 (10 ng to 0.1 pg/reaction) were used. Lane 1: 100 bp ladder; Lane 2: Negative control; Lane 3: 10 ng DNA; Lane 4: 1 ng DNA; Lane 5 100 pg DNA; Lane 6: 10 pg DNA; Lane 7: 1 pg DNA; Lane 8: 0.1 pg DNA).

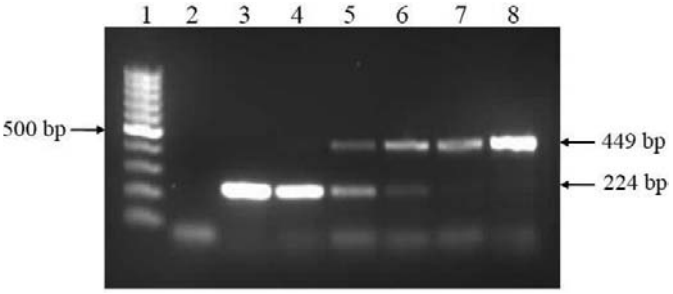

Figure 1. Representative photograph of PCR assay with IAC (1000 copies/reaction) showing detection limit of the assay .

The specificity of PCR assay was tested against the listed bacterial strains (Table 2). The assay could detect all *B. mallei* strains as revealed by presence of a 224-bp *B. mallei* specific amplicon. This amplicon of 224-bp was not observed in other bacterial strains. IAC of 449-bp was observed in all the bacterial strains indicating the successful completion of PCR. With the developed PCR assay, *B. mallei* can be definitively identified and differentiated from *B. pseudomallei* and other closely related species (Table 2).

The developed PCR is robust assay, as there was no significant loss in the visibility of bands at less (20 per cent) or more (20 per cent) concentrations of PCR reagents and IAC DNA. Temperature variation of ±2 °C also did not make any change in PCR profile.

3.3 Validation of PCR Assay with Environmental Samples Artificially Inoculated with *B. mallei*

PCR after 8 h pre-enrichment of sample in glycerol dextrose broth could detect as low as 7.2×10^3 CFU/ml of *B. mallei* in tap water. Further enrichment of sample for 24 h reduced the detection limit of PCR to as low as 72 CFU/ml in tap water. However, when artificially spiked samples of Bengal gram and grass were enriched for 8 h, the detection limit of PCR was 7.2×10^4 CFU/g of *B. mallei*. Neither IAC (449-bp) nor *B. mallei* specific amplicon (224-bp) bands could be detected on agarose gel electrophoresis of PCR products of Bengal gram and grass samples after 24 h of enrichment.

4. DISCUSSION

Rapid, reliable and specific detection of *B. mallei* is essential to start the appropriate antibiotic therapy at an early stage of the disease. Detection of *B. mallei* employing standard microbiological and biochemical tests is time consuming and sometimes requires more than seven days.

Table 2. Bacterial strains used for the evaluation of specificity of Bms primers

Bacterial strain	224-bp Bm specific amplicon	449-bp IAC
Burkholderia mallei		
NCTC 10230	+	+
NCTC 10229	+	+
NCTC 10245	+	+
NCTC 10247	+	+
NCTC 10260	+	+
NCTC 3709	+	+
Burkholderia pseudomallei		
NCTC 4845	–	+
NCTC 10274	–	+
NCTC 13392	–	+
NCTC 6700	–	+
Burkholderia cepacia ATCC 25416	–	+
Burkholderia cenocepacia 7656 DB174BUCE1	–	+
Pseudomonas aeruginosa ATCC 9027	–	+
Pseudomonas fluorescens ATCC 13525	–	+
Pseudomonas putida ATCC 49128	–	+
Brucella abortus NCTC 10093	–	+
Brucella melitensis NCTC 10094	–	+
Bacillus anthracis Sterne	–	+
Bacillus thuringiensis MTCC 4714	–	+
Bacillus subtilis MTCC 736	–	+
Bacillus cereus ATCC 10876	–	+
Vibrio cholerae ATCC 14033	–	+
Staphylococcus aureus DB180STAU66	–	+
Enterococcus faecalis ATCC 29212	–	+
Salmonella		
typhi DB177SAEN1	–	+
virchow MTCC 1163	–	+
typhimurium MTCC 1251	–	+
enteritidis DB179SAEN3	–	+
weltevreden MTCC 1169	–	+
infantis MTCC 1167	–	+
paratyphi A DB178SAEN2	–	+
Escherichia coli MTCC 730	–	+
Klebsiella pneumoniae MTCC 432	–	+
Serratia liquifaciens MTCC 1620	–	+
Shigella flexneri MTCC 1457	–	+
Yersinia enterocolitica DB176YEEN1	–	+

Various molecular techniques for differentiation of *B. mallei* and *B. pseudomallei* are labour intensive and require several hours to complete[12,27]. In contrast, standard PCR techniques reduce labour and require significantly less time to give results. Therefore, PCR, owing to its rapidity and specificity is a method of choice for early detection of *B. mallei*. However, development of PCR-based methodologies for detection and differentiation of *B. mallei* from *B. pseudomallei* have been troublesome due to high level of genetic similarities between various *Burkholderia* species[12-15].

In present study, a 244-bp *B. mallei* specific DNA sequence (between coordinates 376072 and 376315 on + strand of chromosome II of *B. mallei* NCTC 10229) was identified by screening around 3000 ORFs using BLASTn search. Recent BLASTn search revealed the presence of this target sequence in all the ten *B. mallei* strains (*viz.* 092700E, 2000031063, 23344, 6, ATCC 23344, BMQ, FMH 23344, NCTC 10229, NCTC 10247 and SAVP1) whose genomes have been sequenced completely and in twelve other strains (*viz.* GB8, 2002721280, JHU, FMH, ATCC 10399, PRL-20, China 7, China 5, A188, 2000031281, Strain 11 and A 193) of *B. mallei* with partially sequenced genomes. This target sequence was absent in most closely related *B. pseudomallei* strains except one strain *viz. B. pseudomallei* 7894. The 244-bp target sequence exhibited 91 per cent sequence identity with its orthologue in *B. pseudomallei* 7894 strain. Hence, the PCR assay developed in this study may give false positive result with one strain of *B. pseudomallei* i.e. 7894, which is a human isolate obtained from Ecuador in 1962 and does not phylogenetically belong to Asian or Australian populations of *B. pseudomallei*[28]. Hence, the PCR assay can be used for specific detection and differentiation of *B. mallei* from *B. pseudomallei* in Asia, Australia, and other regions where *B. pseudomallei* strain 7894 is not prevalent.

PCR assays are usually performed without internal amplification controls (IACs). In PCR, positive and negative controls generally indicate about the efficacy of the assay, but do not confirm the true negative results of a test sample. An individual test negative results may either be true-negative or false-negative due to the presence of PCR inhibitors within the sample[29]. Therefore, inclusion of IAC in a PCR assay is essential for its validation through a multicentre collaborative trial and IACs in PCR assays would give further assurance of reliable results[30]. In present PCR based assay for detection of *B. mallei*, we incorporated an IAC of 449-bp for detection of PCR failures. Minimum concentration of IAC DNA (1000 copies) that generated a good visible band, was optimised to avoid competition between IAC DNA and target DNA for Bms primers particularly at low concentration of target DNA.

The developed PCR assay was found to be specific for detection of *B. mallei* and its differentiation from *B. pseudomallei*. The amplicon of *B. mallei* specific sequence could be detected in all the six strains of *B. mallei* used in this study. Earlier reported PCR based assay and a real time PCR based assay for specific detection of *B. mallei* targeted $bimA_{Bm}$ gene of *B. mallei*[17, 18]. However, later on this gene was reported in a few other strains of *B. pseudomallei* confirming the non-specificity of $bimA_{Bm}$ gene[20]. Moreover, no IAC was included in the conventional PCR assays for detection of *B. mallei* for reliability of the results[16,23].

Robustness is also an important criterion for the diagnostic PCR. The performance of the present PCR assay was not affected even when 20 per cent less or 20 per cent more concentration of PCR reagents and IAC DNA was used. The assay could withstand an annealing temperature variation of ±2 °C, indicating the robustness of this assay.

Validation of the PCR assay was performed by detecting *B. mallei* in artificially inoculated environmental samples. Water,

Bengal gram and grass were selected for artificial inoculation because these matrices play a role in transmission of glanders in equines. PCR detected *B. mallei* in tap water after 8 h and 24 h of enrichment. The assay could also detect *B. mallei* from Bengal gram and grass after 8 h of enrichment however, PCR failure was observed after 24 h enrichment. Absence of IAC and *B. mallei* specific bands in samples from Bengal gram and grass after 24 h of enrichment indicated the PCR failure. This may be due to accumulation of PCR inhibitors. The PCR inhibitors could have accumulated because of mechanical shearing of Bengal gram/grass after 24 h enrichment and/or because of overgrowth of non-target bacteria. Results of this study showed that glycerol dextrose broth (with 200 Units/ml of penicillin) lose its selectivity after 8 h of incubation. Loss of selectivity after 6 h of enrichment was reported earlier also by Merwyn[31], *et al*.

This investigation describes a *B. mallei*-specific PCR assay with IAC, capable of differentiating this highly infectious pathogen from *B. pseudomallei* and other closely related bacterial species. Results of present study suggest that the PCR assay can be used for identification/detection of *B. mallei* from environmental samples.

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