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ORIGINAL ARTICLE

Evaluation of PCR, DNA hybridization and immunomagnetic separation – PCR for detection of *Burkholderia mallei* in artificially inoculated environmental samples

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Abstract Glanders is highly contagious disease of equines, caused by *Burkholderia mallei*. The disease though rare, can be transmitted to humans. Here, we report a strategy for rapid detection of *B. mallei* from environmental samples. Different bacteriological media were evaluated and brain heart infusion broth medium with selective supplements (BHIB-SS) of penicillin (200 U/ml) and crystal violet (1:10,00000) was found to support the maximum growth of *B. mallei* even in the presence of other bacteria like *Escherichia coli* and *Staphylococcus aureus*. A polymerase chain reaction (PCR) and a DNA hybridization method was standardized for 823 bp specific DNA sequence of *B. mallei*. To enable the quicker and direct enrichment of *B. mallei* bacteria from environmental samples, an immunomagnetic separation (IMS) method was also standardized. Water, husk, grass and gram samples were artificially contaminated by *B. mallei* bacteria and after enrichment of *B. mallei* in BHIB-SS, detection was carried out by PCR and DNA hybridization. PCR was found to be a better method of the two with a detection limit of 10^4–10^6 CFU/ml (6 h enrichment in BHIB-SS) in water and other particulate matrices. Detection by PCR in the above samples without enrichment in BHIB-SS was carried out following IMS where the detection limit was about 1–2 log higher than PCR following enrichment in BHIB-SS. We recommend PCR for 823 bp for detection of *B. mallei* from environmental samples either following enrichment in BHIB-SS or IMS. IMS-PCR method may be preferred in situations where numbers of *B. mallei* bacteria are expected to be high and results are required in short time.

Keywords *Burkholderia mallei* · DNA hybridization · Immunomagnetic separation · PCR · Rapid detection

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Introduction

Glanders is highly contagious disease of equines, caused by *Burkholderia mallei*, a gram negative bacterium. The organism is thought to be an obligate mammalian pathogen with equines serving as the reservoir for infection [1]. Glanders mostly takes a chronic course in horses whereas donkeys and mules die within 4–7 days. The transmission of disease occurs via food and water contaminated by nasal discharge of infected animals [2]. At the turn of the 20th century, glanders was an important cause of death among horses and there were secondary, often fatal infections in human [3]. Presently, the disease has been eradicated from many countries through countermeasures like intensive blood testing, rigorous killing of positive animals and strict trading restrictions [4]. However, glanders is believed to be still endemic in Middle East, Northern Africa and Asia [5]. Outbreaks of glanders have also been reported from India and Pakistan [6, 7].

In humans, glanders is primarily an occupational disease that affects individuals who have close contact with infected

animals, such as veterinarians, grooms and farmers [8]. The acute infection is characterized by necrosis of the tracheobronchial tree, pustular skin lesions and results in either a febrile pneumonia, if the organism was inhaled or signs of sepsis and multiple abscesses, if the skin was the portal of entry. The human infection can be severe and life-threatening and always fatal if untreated or misdiagnosed [3]. No vaccines are available against glanders and little is known about the appropriate antibiotic regimen [9]. As a consequence and also because of the lethal and contagious nature of the disease, *B. mallei* is considered an ideal agent for biological warfare [10].

Laboratory diagnosis of glander cases would have been best possible by bacteriological confirmation but it serves little purpose as glander's bacillus is seldom present in the blood circulation and stays for occasional short bacteremic phase mainly in acute phase. *B. mallei* usually spread via lymphatics. The isolation of bacillus from nasal discharge is not easy as it is usually highly contaminated [11]. Mallein test was developed as field test both for confirmation and for screening of in-contact animals. Several other serological tests were developed which include agglutination test [12], Indirect haemagglutination (IHA) test [13], precipitation test [12], immunodiffusion test [14], counter-immuno-electrophoresis test [15], complement fixation test [16], fluorescent antibody test [17], enzyme-linked immunosor-bent assay (ELISA) [18]. Complement fixation test and ELISA are more sensitive than other tests mentioned above. However, no single test is fully dependable [11]. With the availability of molecular methods like polymerase chain reaction (PCR) for diagnosis of many pathogens, similar methods have also been developed for *B. mallei* [19, 20].

However, none of diagnostic methods has ever been applied to non-clinical samples like water and feed. *B. mallei* bacteria have been reported to survive in the environment for up to 6 weeks and because of transmission of disease can occur via food and water contaminated by nasal discharge of infected animal [2], the present study was undertaken to devise a suitable strategy for detection of *B. mallei* from non-clinical environmental samples. Herein, we report designing of a suitable growth medium for *B. mallei*, especially for environmental samples. Further, we report the development of three molecular methods namely, PCR, DNA hybridization and immunomagnetic separation-PCR (IMS-PCR) for rapid detection of *B. mallei*. These assays were evaluated for detection of *B. mallei* in artificially contaminated environmental samples either directly (IMS-PCR) or with prior selective enrichment in designed selective medium.

Materials and methods

Bacterial strain

Standard strain of *B. mallei* (NCTC 10247), was obtained from CMVL, Meerut. Cultures of *Escherichia coli*, *Salmonella typhi, Pseudomonas aeruginosa, Brucella abortus* and *Staphylococcus aureus* were obtained from the laboratory collection.

Selection of suitable bacteriological medium

Three different media, e.g. glycerol beef extract broth [10 g peptone, 5 g sodium chloride, 3 g beef extract, 40 ml glycerol, 1,000 ml double distilled water (DDW), pH 7.4 ± 0.2], brain heart infusion broth (BHIB, Difco) and glycerol dextrose broth (10 g peptone, 5 g beef extract, 5 g sodium chloride, 10 g dextrose, 40 ml glycerol, 1,000 ml DDW, pH 7.4 ± 0.2) were initially used to compare the growth of *B. mallei* bacteria. The media were inoculated and were incubated at 37°C in incubator shaker (180 rpm). Samples (1 ml) were drawn after 6 h of incubation and growth of bacterial strains was determined by spread plate technique onto glycerol dextrose agar (GDA). The fold increase in growth of *B. mallei* in different media was taken as the criterion for selection of one medium.

To make the medium selective for growth of *B. mallei*, different antibiotics like penicillin, bacitricin, polymyxin B and non-antibiotic antibacterial agent like crystal violet were used. A known number of cells from overnight grown culture of *B. mallei*, *E. coli* and *S. aureus* were inoculated into BHIB having different working concentrations of antibiotic combination [penicillin (50 U, 100 U, 200 U/ml) + crystal violet 1:10,00,000], [polymyxin B (10 U/ml) + bacitracin (12.5 U/ml)] and incubated at 37°C in incubator shaker. Samples were drawn at 6 and 24 h of incubation and spread plated onto GDA. The growth of *E. coli* was also determined using MacConkey agar. The selective enrichment capabilities were compared among the antibiotic combinations and a suitable combination and concentration were chosen for further studies.

Polymerase chain reaction

Genomic DNA was extracted from bacterial cultures either by boiling method [21] or by using Promega genomic DNA purification kit. A set of primers (BM-4 - 5′ CGATCCTGGTGTGCTCGGCCG 3′ and Bm-5 - 5′ CGCAGACCTTCTTCCATCGCGATC 3′) targeting the 823 bp gene of *B. mallei* [22] were designed, synthesized by Operon Biotechnologies, Germany, and used for PCR.

The DNA amplification was accomplished in presence of 50 ng of template DNA, 1.0 μM of each primer and 1.25 U of Taq DNA polymerase, 200 μM dNTP, 1.5 mM $MgCl_2$ in 1 × PCR buffer, in final volume of 25 μl. The reaction mixture was denatured for 5 min at 95°C in the beginning, followed by 35 cycles each comprising of the three segments of at 95°C for 1 min, 59°C for 1 min and 72°C for 2 min. This was followed by 1 cycle at 72°C for 10 min. The reaction mixture was subjected to electrophoresis on 1.2% agarose gel to analyze the PCR product. The detection limit of optimized PCR was determined by amplifying the serially diluted DNA derived from *B. mallei*. The specificity of primers to 823 bp gene specific to *B. mallei* was examined by carrying out PCR along with genomic DNA of other bacterial strains as mentioned earlier.

DNA hybridization

Different DNA probes targeting either full 823 bp gene or BM-4 primer were used in the study. The 823 bp gene of *B. mallei* amplified by PCR or primer BM-4 were labeled with digoxigenin as per the protocol supplied by the manufacturer of Roche - DIG DNA – labeling and detection kit. The primer of BM-4 was labeled with biotin through commercial services of Qiagen, Germany. The DNA hybridization technique was standardized as per the protocol supplied by the manufacturer in Roche - DIG DNA – labeling and detection kit. Briefly, 1 μl of DNA template denatured for 10 min in a boiling water bath was spotted onto the positively charged nylon membrane (Millipore, Bedford, USA). The DNA was fixed onto the membrane by baking at 80°C for 2 h and blocked for 30 min with the blocking solution provided in the kit. Hybridization was carried out overnight at 68°C with labeled probe in hybridization buffer (0.1% N-Lauryl sarcosine, 0.02% SDS, 1% blocking reagent, 5× SSC). Stringent washing was carried out with wash buffer-1 (2 × SSC, 0.1% SDS) twice and wash buffer-2 (0.5 × SSC, 0.1% SDS). Washed membrane was incubated with 1:5,000 dilution of antidigoxigenin-AP antibody conjugate for DIG labeled probes and 1:1,000 dilution of streptavidin-AP antibody conjugate for biotin labeled probe, prepared in blocking solution for 30 min. The membrane was washed with washing buffer-3 (0.1 M maleic acid, 0.15 M sodium chloride, pH 7.5, 0.3% (v/v) tween 20), and further equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M sodium chloride, pH 9.5). Later, the membrane was developed with substrate NBT/BCIP (200 μl of NBT/BCIP substrate in 10 ml of detection buffer) by keeping in the dark for 30 min. In all hybridization experiments sperm DNA acted as a negative control. Detection limit of different probes was determined by serially diluting the known amount of target DNA and using them for hybridization. Specificity of different probes was evaluated with the DNA extracted from different organisms.

Preparation of antibodies to whole cell lysate (WCL) antigen

B. mallei was grown in large quantity in glycerol dextrose broth and harvested by centrifugation at 8,000 g for 15 min at 4°C. The cells were washed with and suspended in small volume of phosphate buffer saline (PBS), sonicated and centrifuged at 12,000 g for 10 min at 4°C. The supernatant was collected and protein content was estimated by method of Lowry et al. [23]. Antiserum to WCL antigen was raised in New Zealand white rabbits according to the standard method [24]. The antibody titer was determined by Dot-ELISA [25]. The antiserum (10 ml) was precipitated by saturated ammonium sulfate solution and dialyzed against PBS. The ammonium sulfate precipitated γ-globulins were fractionated by gel exclusion chromatography using Bio-Rad matrix Bio A1.5. The immunoglobulin G (IgG) fraction was collected, concentrated and protein content was estimated.

Immunomagnetic separation - polymerase chain reaction

IgG fraction as purified above was coated onto epoxy-coated magnetic beads (Dynal Biotech, Oslo, Norway, 2.8 μm dia.) according to previously reported protocol [21]. To determine the optimum concentration of coating antibody, various concentrations (5, 10, 15 μg) of IgG fraction was coated onto per milligram of magnetic beads. A known number of test organism, *B. mallei*, was captured onto antibody coated magnetic beads by incubating the organism with the beads on a rotatory shaker at room temperature for 1 h, and washed with minimum recovery diluent (MRD, 1/10 dilution of BHI in 0.1 M PB). The beads were concentrated using magnet (MPL1, Dynal), re-suspended and serially diluted in MRD. The bacterial count was determined by plate count method. The antibody concentration on the beads which captured maximum number of *B. mallei* was selected by comparing the colony forming units (CFU) formed before and after capturing.

For IMS of *B. mallei* by the coated beads, 1 ml of bacterial culture was mixed with 10 μl (10^7) beads. After mixing for 1 h in Dynal mixer, the beads were washed with MRD (1/10 dilution of BHI) twice followed by concentration by magnet (MPL1, Dynal). The beads were suspended in 50 μl of distilled water (DW) and boiled for 10 min. The supernatant was separated and an aliquot of 2 μl was used as template DNA for 25 μl PCR reaction.

Environmental samples and artificial inoculation with *B. mallei*

Duplicate samples of municipal water, tap water, and aqua guard water (100 ml each) were collected from municipal water supply and from Microbiology Division of this establishment in sterile containers. Grass, husk and gram were collected from local market. Serially 10-fold diluted overnight grown *B. mallei* culture (1 ml) was spiked in 9.0 ml of water, 9.0 g of Bengal gram (soaked overnight in sterile DDW), or 1.0 g of each of grass or husk samples in such a way that different aliquot of each sample received *B. mallei* bacteria from 10^0–10^7 through 10^1, 10^2, 10^3, 10^4, 10^5, 10^6 CFU/ml or g. After keeping at room temperature for 20 min, 90 ml of BHIB having selective supplements (BHIB-SS) [penicillin (200 U/ml) and crystal violet (1:1,000,000)] was added to each sample and incubated at 37°C in a incubator shaker for 24 h. One milliliter sample was collected from spiked material after 6 and 24 h of growth and DNA was extracted in 50 µl of DW by boiling method, of which 1 or 2 µl was used as template DNA for detection by PCR and DNA hybridization, respectively. The samples were processed directly for IMS-PCR after 20 min without any incubation in BHIB-SS medium. One milliliter sample was collected 20 min after addition of BHIB-SS medium and processed as described in IMS-PCR section. The exact number of spiked bacteria was determined by plate count method.

Results and discussion

Rapid detection of *B. mallei* in environmental samples is absolutely important particularly with the weaponization potential of the organism. The standard laboratory [11] and more recently developed molecular methods [19, 20] have targeted the clinical samples only; however, the fact that *B. mallei* can survive in environmental samples and transmission of disease can occur via food and water contaminated by nasal discharge of the infected animal, the present work was aimed to design effective strategy for rapid detection of *B. mallei* from environmental samples.

The environmental samples can be contaminated with various other non-target bacteria; therefore, we first undertook the work of finding a suitable bacteriological medium that can selectively enrich *B. mallei*. Two additional bacterial species, one representing gram negative bacteria (*E. coli*) and other representing gram positive bacteria (*S. aureus*) were included as non-target bacteria. Of the three media evaluated in the study for growth of *B. mallei*, maximum fold (185-fold) increase in growth was observed in BHIB. The second best medium supporting the growth was found to be glycerol dextrose broth where 90-fold increase in growth of *B. mallei* was observed. When *B. mallei* was grown in combination with other bacterial strain (*S. aureus* or *E. coli*), the BHIB supported highest growth (300-fold increase) for *B. mallei* followed by glycerol beef extract broth. *E. coli* grew well in all media and at times it was difficult to count the colonies of other two species. More consistent results were observed in BHIB than glycerol dextrose broth and also because the former medium yielded better growth of *B. mallei* even in the presence of *E. coli*, BHIB was selected for further studies (Table 1).

For selective enrichment of *B. mallei* in presence of *E. coli* and *S. aureus*, different antibiotics and crystal violet were evaluated (Table 2). The combination of penicillin and crystal violet (penicillin 100 or 200 U/ml + crystal violet 1:1,000,000) suppressed the growth of other bacteria and allowed the growth of *B. mallei* either after 6 h enrichment or 24 h enrichment. The combination of penicillin (200 U/ml) and crystal violet (1:1,000,000) was used with BHIB (now named as BHIB-SS) for further experiments. Combination of penicillin and crystal violet with penicillin concentration of 50 U or less (30, 20) (data

Table 1 Fold increase in number of bacteria in various bacteriological media

Inoculating bacteria	Fold increase [mean value (range)] of various bacteria after 6 h of incubation		
	Glycerol beef extract broth	Glycerol dextrose broth	Brain heart infusion broth
B. mallei	77 (45–130)	90 (10–180)	185 (95–300)
B. mallei +	30	40	45
S. aureus	159	120	110
B. mallei +	65	35	45
E. coli	261	1,270	2,750
B. mallei +	175	150	300
S. aureus +	205	220	200
E. coli	1,560	1,750	1,200

Inoculum size (single culture experiment): *B. mallei* - 3–20 CFU/ml.
Inoculum size (mixed culture experiment): *B. mallei* - 20 CFU/ml, *E. coli* - 20 CFU/ml, *S. aureus* - 20 CFU/ml. Values of one representative experiment from minimum three experiments are shown.

Table 2 Effect of selective agents on the growth of *B. mallei* and other bacteria

Organism	Selective agents (concentration)			
	Penicillin (50 U/mL) + C.V (1:10,00000)	Penicillin (100 U/ml) + CV (1:1,000,000))	Penicillin (200 U/ml) + CV (1:1,000,000)	Polymyxin B (10 U/ml) + bacitricin (12.5 U/ml)
Number of CFU after 6 h of incubation at 37°C under shaking conditions				
B. mallei	1.0×10^3	1.8×10^3	1.7×10^3	1.1×10^2
E. coli	1.2×10^2	No growth	No growth	No growth
S. aureus	No growth	No growth	No growth	No growth
Number of CFU after 24 h of incubation at 37°C under shaking conditions				
B. mallei	Not detectable	5.8×10^7	5.6×10^7	2.0×10^4
E. coli	1.2×10^7	No growth	No growth	No growth
S. aureus	Not detectable	No growth	No growth	No growth

Inoculam size: *B. mallei* - 36 CFU/ml, *E. coli* - 53 CFU/ml, *S. aureus* - 40 CFU/ml. Values of one representative experiment from minimum two experiments are shown.

not shown) was unable to limit the growth of *E. coli*. The combination of polymyxin B and bacitracin was found to be inhibitory to the growth of *B. mallei*.

PCR was optimized for 823 bp DNA sequence (Fig. 1). The detection limit of PCR was found to be 500 pg. The PCR primers were checked for specificity and no cross-reaction was observed with *E. coli*, *S. typhi*, *P. aeruginosa, B. abortus* and *S. aureus*.

To select a suitable DNA probe having better sensitivity and specificity toward 823 bp DNA sequence specific to *B. mallei*, three different probes were used for DNA hybridization. DIG labeled and biotin labeled BM-4 were found specific, whereas, DIG labeled 823 bp gene showed cross-reactivity with other bacteria. Most probable reason for non-specificity of DIG labeled 823 bp probes is its size which results in non-specific binding to genomic DNA of other bacteria. As expected BM-4, being a shorter sequence was found specific. The detection limit and optimum concentration required for hybridization for DIG labeled BM-4 and biotin labeled BM-4 was found to be 1 ng and 50 ng and, 200 ng/ml and 4,000 ng/ml, respectively. Hence, DIG labeled BM-4 was chosen for further experiments.

The antiserum raised against WCL antigen of *B. mallei* had the titer of >1:128,000. The IgG was purified from the antiserum. For optimization of IMS-PCR, different concentrations of IgG antibody (5–15 µg/mg of beads) were coated onto the beads for capturing the *B. mallei*. It was observed that 10 µg IgG/mg of beads had better capturing (enrichment) efficiency (37.83%), than any other concentration (data not shown). The detection limit of IMS-PCR was same as that of PCR.

Water, husk, Bengal gram and grass samples are closely associated with equines, therefore these samples were chosen as environmental samples for artificial inoculation with *B. mallei*. After artificial inoculation, BHIB-SS was added to the samples. The samples were incubated at 37°C under shaking conditions and *B. mallei* bacteria were detected by PCR and DNA hybridization after 6 or 24 h. The results of detection limits of various test formats (BHIB-SS 6 h - PCR, BHIB-SS 24 h - PCR, BHIB -SS 6 h - DNA hybridization, BHIB-SS 24 h - DNA hybridization) in different environmental samples are shown in Table 3. PCR with prior 6 h enrichment in BHIB-SS was found to be the most sensitive assay with detection limit of about 10^4 bacteria/ml in water (Fig. 1). The detection limit of this assay was, however, one or two log higher in particulate matrices. As low as 31 bacteria could be detected by PCR in municipal water after 24 h of enrichment in BHIB-SS. *B. mallei* could not be detected in grass and gram samples either by PCR or DNA hybridization after 24 h of incubation. This may be due to the overgrowth of non-target bacteria, which might have masked the growth of *B. mallei*. It seems that BHIB-SS looses its selectivity after 6 h of incubation. *B. mallei*

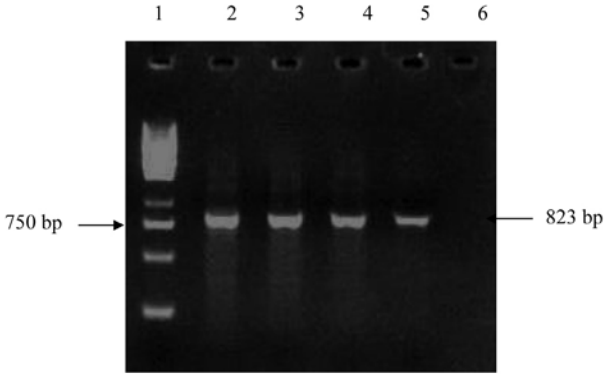

Fig. 1 Detection of *B. mallei* in artificially inoculated municipal water sample by PCR after 6 h of enrichment in BHIB-SS. Lane 1: 1 kb Ladder; Lane 2: Positive control; Lane 3–6: 3.1×10^6 to 3.1×10^3 CFU/ml.

Table 3 Determination of detection limit of various molecular methods in artificially inoculated environmental samples with *B. mallei*

Samples	PCR		DNA hybridization		IMS-PCR
	6 h	24 h	6 h	24 h	20 min*
Aqua guard water	9.8×10^4	9.8×10^1	3.7×10^5	3.7×10^1	1.3×10^6
Tap water	9.8×10^4	9.8×10^1	3.7×10^5	3.7×10^1	1.3×10^6
Municipal water	3.1×10^4	3.1×10^1	3.7×10^5	3.7×10^1	3.5×10^6
Grass	1.4×10^6	ND	5.6×10^7	ND	4.3×10^7
Gram	5.6×10^5	ND	2.1×10^7	ND	4.3×10^7
Husk	1.0×10^6	1.0×10^4	4.8×10^7	4.8×10^6	7.1×10^7

The number represents the detection limit in CFU in 1 ml or 1 g of the sample.
*Detection of *B. mallei* by IMS-PCR was carried out 20 min after artificial inoculation.
ND = Not detectable.

could be detected in husk after 24 h of enrichment both by PCR and DNA hybridization. Husk being a dry material, probably, did not have much contaminating bacteria; hence, *B. mallei* could grow in sufficient numbers. The detection limit of DNA hybridization method was generally one log higher than PCR (Fig. 2). Detection by PCR in above samples without enrichment in BHIB-SS was carried out following IMS (IMS-PCR). IMS was done immediately (20 min) after addition of the BHIB-SS to the environmental sample. *B. mallei* of order of 10^6–10^7 CFU/ml could be detected by IMS-PCR. Though, the IMS-PCR procedure is less sensitive than PCR or DNA hybridization with prior enrichment in BHIB-SS for 6 h, yet, the method is quicker by at least 4 h.

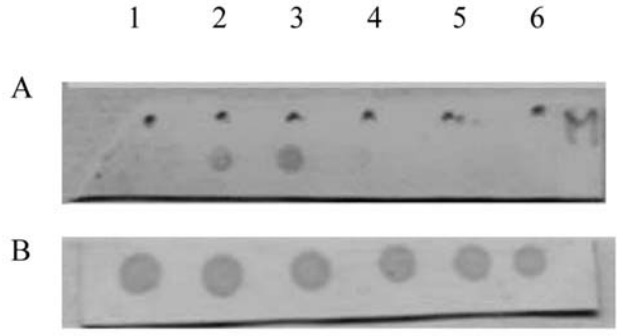

Fig. 2 Detection of *B. mallei* in artificially inoculated municipal water sample by DNA hybridization after 6 h (Panel A) or 24 h (Panel B) of enrichment in BHIB-SS. Panel A: Spot 1: Negative control; Spot 2: Positive control; Spot 3–6: 3.7×10^5 to 3.7×10^2 CFU/ml. Panel B: Spot 1–6: 3.7×10^6 to 3.7×10^1 CFU/ml.

Based upon the results of this study, we suggest that BHIB-SS can be used for selective enrichment of *B. mallei* from environmental samples. Though, BHIB-SS selectively enriched the growth of *B. mallei* in presence of *E. coli* and *S. aureus* yet its true potential could only be determined in artificially inoculated in environmental samples. In these samples, the medium allowed the selective growth of *B. mallei* for 6 h or more, proving its usefulness. After enrichment of 6 h in BHIB-SS, *B. mallei* can be detected by PCR for 823 bp gene. The assay can be completed within one working day (8–10 h) with a detection limit of 10^4–10^6 CFU/ml depending upon the nature of sample matrix. However, in situations where, large numbers of *B. mallei* bacteria are expected, and quicker results are required, IMS-PCR method can be used which can be completed in <6 h.

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