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A sensitive & specific multiplex PCR assay for simultaneous detection of *Bacillus anthracis, Yersinia pestis, Burkholderia pseudomallei* & *Brucella* species

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***Background & objectives*: *Bacillus anthracis, Yersinia pestis, Burkholderia pseudomallei* and *Brucella* species are potential biowarfare agents. Classical bacteriological methods for their identification are cumbersome, time consuming and of potential risk to the handler.**

***Methods*: We describe a sensitive and specific multiplex polymerase chain reaction (mPCR) assay involving novel primers sets for the simultaneous detection of *B. anthracis, Y. pestis, B. pseudomallei* and *Brucella* species. An additional non-competitive internal amplification control (IAC) was also included.**

***Results*: The mPCR was found to be specific when tested against closely related organisms. The sensitivity of the assay in spiked blood samples was 50 colony forming units (cfus)/25 µl reaction, for the detection of *B. anthracis, Y. pestis* and *Brucella* species; and 150 cfus/25 µl reaction, for *B. pseudomallei.* The assay proved useful in correctly and promptly identifing the clinical isolates of the targeted agents recovered from patients, compared to the gold standard culture methods.**

***Interpretation & conclusion*: The assay described in this study showed promise to be useful in application as a routine detection cum diagnostic method for these pathogens.**

Key words Biowarfare - fever of unknown origin - internal amplification control - multiplex PCR - spiking

Bacillus anthracis, Yersinia pestis, Burkholderia pseudomallei and *Brucella* species are all listed by the Centers for Disease Control and Prevention (CDC) in 'Category A' and 'Category B', based on their ability to be used as bioterrorism agents and to cause disease in general. *B. anthracis* and *Y. pestis* are listed under Category A, which can be easily disseminated or transmitted from person to person causing high mortality rates and possessing the potential for major public health impact. *B. pseudomallei* and *Brucella* species are under Category B, easy to disseminate and result in moderate morbidity and low mortality rates[1]. Except for *B. anthracis*, the other three organisms also act as causative agents for infectious

fevers of unknown origin (FUO), but are not routinely investigated. Despite recent advances in diagnostic techniques, FUO still remain a formidable diagnostic challenge[2]. Thus, rapid and reliable detection of these organisms is imperative for prompt and efficient patient management and disease control. For the identification of these organisms, generally direct methods based on bacteriological isolation are applied, which are cumbersome, time consuming and a potential threat to the handler. PCR is a useful approach for the diagnosis of bacterial infections, not detectable by classical bacteriology. Several PCR based molecular diagnostics in uniplex reactions have already been reported for these organisms[3-6]. Multiplex PCR (mPCR) first described by Chamberlain *et al* in 1988[7] is a useful tool for the identification of bacteria as well as other organisms[8-12] with the possibility of detecting multiple organisms in a single tube reaction.

Here we describe a sensitive and specific mPCR involving novel primer sets for the simultaneous detection of *B. anthracis, Y. pestis, B. pseudomallei* and *Brucella* species. An additional 16S rRNA universal primer set was also added to the reaction as a non-competitive internal amplification control (IAC). The specificity of this mPCR was evaluated with closely related organisms and its sensitivity for its application as a routine diagnostic method, was evaluated on spiked blood samples.

Material & Methods

The study was carried out in Microbiology Division of Defence Research & Development Establishment (DRDE), Gwalior, India, during March 2010 to September 2010.

Standard bacterial strains, clinical isolates and culture conditions: Standard bacterial strains used in the study are shown in Table I and the clinical isolates in Table II. These organisms were grown overnight at 37°C on brain heart infusion (BHI) agar/broth (Difco Laboratories, USA).

Oligonucleotide primers: The sequences of primers used in the study are listed in Table III. Specific primers for the *pag* gene in *B. anthracis*[13], *caf1* gene in *Y. pestis*[16] and 16s rRNA gene for Universal IAC[15] were obtained from published literature, while two sets of primers targeting the IS711 gene in *Brucella* species and the *fur* gene in *B. pseudomallei*, respectively were self-designed. All the primer sets were designed using the Gene Runner software (*www.generunner.com*) and obtained from Operon Biotechnologies, Cologne, Germany.

Table I. Bacterial strains used to evaluate the mPCR specificity

Sl. No.	Name of organism	Origin*
1.	*Bacillus anthracis* Sterne strain	IVPM
2.	*B. cereus*	ATCC 13061
3.	*B. sphaericus*	ATCC 4525
4.	*B. circulans*	ATCC 61
5.	*B. licheniformis*	ATCC 12759
6.	*B. thuringiensis*	ATCC 10792
7.	*B. megaterium*	ATCC 14581
8.	*Brucella abortus* S19	IVRI
9.	*Burkholderia pseudomallei*	NCTC 01688
10.	*Escherichia coli*	ATCC 11775
11.	*Yersinia pestis*	A1122, WHO Collaborating Center at CDC
12.	*Y. pseudotuberculosis*	1A, WHO Collaborating Center at CDC
13.	*Y. intermedia*	Norway
14.	*Y. frederiksenii*	Norway
15.	*Y. kristensenii*	Norway
16.	*Y. enterocolitica*	ATCC 23715
17.	*Burkholderia mallei*	NCTC 10260
18.	*Pseudomonas aeruginosa*	IMTECH
19.	*P. mendocina*	IMTECH
20.	*P. pseudoalcaligenes*	IMTECH
21.	*P. alcaligenes*	IMTECH
22.	*Salmonella* Typhi	MTCC 734
23.	*Brucella mellitensis* 16M	IVRI

*ATCC, American Type Culture Collection, Manassas, Va; MTCC, Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chennai, India; NCTC, National Collection of Type Cultures, U.K; IVPM, Institute of Veterinary and Preventive Medicine, Vellore, India; IVRI, Indian Veterinary Research Institute, Izatnagar, Bareilly, India

DNA isolation: Genomic DNA from Gram-negative and Gram-positive bacterial cultures was isolated using Dneasy Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration of the extracted DNA was measured using Smartspec Plus spectrophotometer (Bio-Rad Lab, USA). The purity

Table II. List of clinical isolates used in the study

Clinical isolates*	Origin
Yersinia pestis	1994 Plague outbreak, Surat, Gujarat, India
Bacillus anthracis	Christian Medical College, Vellore, Tamil Nadu, India
Burkholderia pseudomallei	Calicut Medical College, Calicut, Kerala, India
Brucella melintensis	SDM College of Medical Sciences and Hospitals, Dharwad, Karnataka, India

*Eight isolates of each pathogen were used in the study

of the genomic DNA used in the study was within the acceptable range of 1.8-2.0 A_{260}/A_{280}.

DNA amplification: The primers chosen for developing mPCR were initially tested in uniplex PCRs. For each of the uniplex analysis, PCR was performed[17] using MBI (Fermentas, Germany) reagents. Each 25 µl reaction mixture consisted of 10 pmol of the primers, 1x Dream Taq buffer (2mM $MgCl_2$), 0.2 mM dNTP mix, Template DNA (50-70 ng/µl) and 1.2 units of Dream Taq Polymerase enzyme. PCRs were run using the following steps: initial denaturation for four min at 95°C, and 30 cycles of 95°C denaturation temperature for one min, annealing gradient temperature ranging from 50 to 60°C for one min and two min of 72°C extension temperature followed by another five min of final extension temperature at 72°C.

mPCR was performed employing Multiplex PCR Kit (Qiagen). Appropriate adjustments were made in the concentration of critical reagents and thermocycling conditions including reaction volume, template DNA concentration, primer concentration and annealing temperature to obtain optimal amplification of targeted genes. A 20 µl template DNA mix containing 2 µl (100 ng) DNA from each of the chosen organisms was prepared. Similarly, a primer mixture containing all the five primer pairs was made by mixing 10 µl (10 pmol/µl) of each of the forward and reverse primers. A 25 µl mPCR reaction volume contained 4 µl of template DNA mix (20ng of each organism DNA), 0.5-3 µl of primer mix (1 pmol), mPCR master mix and RNAse-Free Water (Qiagen Multiplex PCR Kit). The mPCR consisted of: initial denaturation at 94°C for four min, and 30 amplification cycles constituting denaturation for one min at 94°C, annealing at 50-55°C for one min and extension at 72°C for two min followed by 10 min of final extension at 72°C for incompletely synthesized DNA. The mPCR products were visualized in 2 per cent agarose (Amresco, USA) gels stained with ethidium bromide (0.5 µg/ml), after electrophoresis in a 0.5 X TBE buffer. DNA ladder of 100 bp (Fermentas) was used as size standard.

Evaluation of mPCR: The specificity of the multiplex PCR was evaluated for its cross-reactivity with closely related organisms (Table I). In order to determine the sensitivity, based on colony forming units (cfu) was determined[18] on BHI agar from cultures of *B. anthracis, Y. pestis, B. melitensis* and *B. pseudomallei* and was

Table III. Oligonucleotide primers used in the study

Organism	Target gene	Sequence 5'-3'	Product size (bp)	Reference
Bacillus anthracis	*Pag*	F - TCC TAA CAC TAA CGA AGT GAA GGA R- CTG GTA GAA GGA TAT ACG GT	596	13
Yersinia pestis	*caf1*	F- ATA CTG CAG ATG AAA AAA ATC AGT TCC R- ATA AAG CTT TTA TTG GTT AGA TAC GGT	517	14
Burkholderia pseudomallei	*Fur*	F- GGA AGC TTA GCC ATG ACC AAT R- TCC TGC AGG TGC CGT ACA TC	428	Developed in the present work
Brucella sp.	IS711	F- GGT TGT TAA AGG AGA ACA GCA R- GAC GAT AGC GTT TCA ACT TG	660	Developed in the present work
Universal IAC primers	16S rRNA	F-AGA GTT TGA TCC TGG CTC AG R-ACG GCT ACC TTG TTA CGA CTT	1500	15

IAC, internal amplification control

spiked into one ml anti-coagulant human blood of healthy volunteers drawn intravenously at concentration starting from 10^6 to 10^1 cfu/ml. DNA was then extracted from the spiked blood using the Dneasy Tissue kit (Qiagen, Hiden, Germany) following manufacturer's instructions for use in mPCR. Following optimization of the mPCR assay, its efficiency was evaluated on eight clinical isolates of each of the pathogens (Table II) and compared with gold standard culture methods.

Results

Multiplex PCR: The concentration of primers was optimized after standardizing on individual PCRs with their respective genes. All the chosen primers gave optimum amplification at concentrations ranging from 10-15 pmol. Gradient annealing temperature studies revealed satisfactory amplification for all the primers at 50, 52 and 55°C. Primers targeting specific genes in *B. anthracis* and *Y. pestis* gave optimum amplification up to 60°C, while in *B. pseudomallei* and *Y. pestis* the band intensity began to diminish after 55°C. Hence, for the mPCR gradient temperatures ranging from 50 to 55°C were tested and amplification at 52°C was found to be optimum. The primer concentrations tested in the mPCR yielded optimum amplification at 10-fold lower concentrations than the uniplex setups. The 16S rRNA universal non-competitive IAC co-amplified with the target DNA and had the amplicon size of 1.5 kb. The standardized mPCR revealed the presence of five amplicons after agarose gel electrophoresis (428 bp for *B. pseudomallei*, 517 bp for *Y. pestis*, 596 bp for *B. anthracis*, 660 bp for *Brucella* species and 1.5 kb for IAC) (Figure).

Evaluation of mPCR assay: The mPCR was checked for its cross-reactivity with DNA of closely related organisms (Table I). No amplified product except for IAC was observed with DNA of other organisms, even on repeated tests (Figure). This showed that the developed mPCR assay had 100 per cent specificity for the chosen organisms. Spiking studies revealed a sensitivity of 50 cfus/25 µl reaction, for the detection of *B. anthracis, Y. pestis* and *Brucella* species; and 150 cfus/25 µl reaction, for the detection of *B. pseudomallei.* The mPCR assay when performed on the clinical isolates could selectively amplify the targeted genes and thus rapidly detect the chosen pathogens.

Discussion

Appearance of clinical symptoms with any of the acute systemic bacterial disease is inadvertently associated with bacteremia. Thus, at the initial appearance of clinical symptoms, the presence of bacteria in the blood can be effectively detected by PCRs. However, the use of uniplex PCR in the diagnostic laboratories is limited in view of cost and the availability of adequate test sample which can be obviated by the mPCR approach[19-22]. Considerable sensitivity has been reported in uniplex PCRs targeting *fur* gene in *B. pseudomallei*, *pag* gene in *B. anthracis*, IS711 element in *Brucella* species and *caf1* in *Y. pestis*[14,23-25]. Using reported primers (for *pag, caf1, 16S rRNA* genes) and

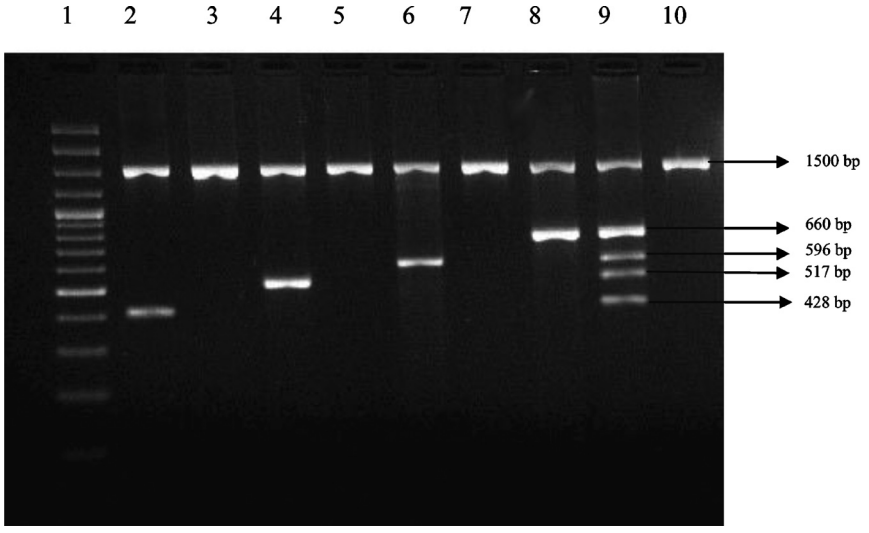

Fig. Cross-reactivity testing of the mPCR with closely related organisms. Lane 1: 100 bp DNA ladder, Lane 2: *Burkholderia pseudomallei*, Lane 3: *Burkholderia mallei*, Lane 4: *Y. pestis*, Lane 5: *Y. pseudotuberculosis*, Lane 6: *Bacillus anthracis*, Lane 7: *Bacillus cereus*, Lane 8: *Brucella mellitus*, Lane 9: Multiplex positive control. Lane 10: *Y. enterocolitica.*

by designing new primers (for IS711 and *fur* genes), a novel mPCR was developed in the present study. It was optimized to a common annealing temperature, maintaining a minimum 80 bp difference between the amplified products for better readability and resolution in the agarose gel. In order to make this mPCR assay acceptable to the present norms of a diagnostic PCR[26], a non-competitive IAC primer targeting the 16S rRNA universal bacterial gene was also added as a positive control. The mPCR system besides conferring enhanced applicability requires additional careful optimizations. The inclusion of additional primer sets in the reaction invariably results in a higher probability of mis-priming and primer dimerization. In addition to effective primer design and selection, annealing temperature standardization, Mg^{2+} ion optimization and maintenance of adequate primer-template DNA ratio is necessary. Further, the use of Hot start PCR eliminates non-specific reactions caused by primer annealing at low temperatures (4-25°C) before the commencement of thermocycling[27]. Hot start PCR was applied in the present study to eliminate non-specific reactions after fulfilling the other optimization requirements.

All the genes targeted in this reaction are established virulence associated factors in each of the organisms. The protective antigen (part of the tripartite exotoxin complex) gene in *B. anthracis* has earlier been used in the diagnosis of anthrax[13]. In the current study, primers targeting the *pag* gene were optimized to mPCR setup and applied as reliable markers for the detection of *B. anthracis*. The IS711 insertion sequence element is exclusively present in the genome of all described species of the genus *Brucella*[28] and the gene sequence was thus used to design specific primers. The fraction 1 (*caf1*) antigen, a major component of the surface of *Y. pestis*[29], encoded by f1 (110) kb plasmid has been classically used for diagnosis of *Y. pestis* infections[16]. The gene is highly conserved and was tried in the present study to provide the characteristic signature of the organism. The ferric uptake regulator (FUR) transcription factor plays an important role in the pathogenesis of *B. pseudomallei*[30]. Specific primers targeting this transcription factor were designed and evaluated for cross-reactivity with other closely related organisms namely, *B. mallei* and *Pseudomonas aeruginosa* in uniplex setup (data not shown). However, the primer sequences utilized in the present study showed homology with other sequences in blast search. In PCR/mPCR, these yielded specific amplification product with the DNA of targeted organism. The specificity of the designed mPCR was evaluated by testing the primer sets against DNA from closely related organisms.

Fever of unknown origin have always been a diagnostic challenge. Routine diagnosis of a suspected FUO based infection evaluates the occurrence of common pathogens causing malaria, typhoid, dengue fever, leptospirosis *etc*. In an earlier study[31], brucellosis was found to be the second most common causative agent of infectious FUO. Human brucellosis is a significant public health problem in India[15]. The occurrences of the other organisms detected in this study as causative agents of FUO are not routinely inspected in diagnostic laboratories. These organisms although rare, have the ability to cause significant damage to public health. The designed mPCR would also find utility in finding the source of the outbreak from among other available environmental samples in such conditions. This would render response teams in mitigating the spread of the disease and consequently aide management and control strategies. The reaction is easy to perform and requires basic PCR apparatus and the mPCR reagents kit. In conclusion, the mPCR described in this study appears to have potential to be used for diagnosis of these potentially harmful pathogens in routine practice.

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