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ORIGINAL ARTICLE

Reverse Line Blot Macroarray for Simultaneous Detection and Characterization of Four Biological Warfare Agents

Vanlalhmuaka · Kulanthaivel Thavachelvam · Urmil Tuteja · Kumari Sarika · Suryanarayana Nagendra · Subodh Kumar

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Abstract The need for a rapid detection and characterization of biowarfare (BW) agents cannot be over emphasized. With diverse array of potential BW pathogen available presently, rapid identification of the pathogen is crucial, so that specific therapy and control measures can be initiated. We have developed a multiplex polymerase chain reaction based reverse line blot macroarray to simultaneously detect four pathogens of BW importance viz. *Bacillus anthracis*, *Yersinia pestis*, *Brucella melitensis* and *Burkholderia pseudomallei*. The multiplex PCR utilizes 14 pairs of primers targeting 18 specific markers. These markers include genes which are genus specific, species-specific chromosomal sequences and virulence markers of plasmid origin. The assay was evaluated on various human, environment and animal isolates. The assay w successful in simultaneous detection and characterization of isolates of the four pathogens on as a single platform with sensitivity ranging from 0.3 pg to 0.3 ng of genomic DNA. The assay was able to detect 5×10^2 cfu/ml for *B. anthracis*, 8×10^2 cfu/ml for *Yersinia* sp., 1.4×10^2 cfu/ml for *B. melitensis* and 4×10^2 cfu/ml for *B. pseudomallei*.

Keywords Reverse line blot · *Bacillus anthracis* · *Yersinia pestis* · *Brucella* · *Burkholderia* · Biowarfare

Introduction

Incidence of anthrax spore attack in the US and the discussions that ensued has fuelled great interests in the need to develop a rapid, sensitive and robust multiplex assays for screening biological warfare (BW) agents. The advantage of multiplex molecular assays over the classical method of isolation and culturing is the ability of the former to characterize the virulence of the identified species simultaneously on a single platform. A number of reports on the usage of real-time PCR assay for simultaneous detection of multiple targets are available [1]. Presently, the drawback of the real-time PCR assay for multiple target detection is the limited number of dyes and detector available commercially. Microarray based multiplex detection assays has the advantage of screening large number of targets on a single platform [2]. However, the complication in putting up a microarray assay, the need for qualified trained person and the cost involved makes the assay unsuitable for small health centres and low-funded laboratories.

Multiplex PCR based reverse line blot hybridization assay (mPCR/RLB) is a DNA hybridization assay where labelled products of multiplex PCR are allowed to hybridize with membrane bound amine modified target probes. The assay has been used for characterization and genotyping study on a number of organisms [3, 4]. In this paper, we describe the development of a much simpler; less expensive mPCR/RLB assay to identify and characterize simultaneously four potential bioweapons classified pathogens, *Bacillus anthracis*, *Yersinia pestis*, *Brucella melitensis* and *Burkholderia pseudomallei.*

Materials and Methods

Bacterial Isolates

The pathogens included in the present study consist of both field isolates and standard strains along with their closely

Vanlalhmuaka (✉) · K. Thavachelvam · U. Tuteja · K. Sarika · S. Nagendra · S. Kumar
Division of Microbiology, Defence Research and Development Establishment, Jhansi Road, Gwalior 474002, India
e-mail: vanlalh@gmail.com

related species (Table 1). Commonly encountered pathogens were also included for specificity studies. All culturing and propagation of the organisms was done using brain heart infusion broth and agar (Difco, MD, USA) at 37 °C for 12–14 h.

Primers and Probes

The probes consist of 5′-terminal amino modified oligonucleotides with size ranging from 20 to 30 bases. They were designed to have similar melting temperature of approximately 60 °C. For mPCR, 14 pairs of primers, 20–30 bases in length with 5′-terminal biotin modified, were designed with similar melting temperature. The primers were designed in such a way that the amplicon size does not exceed 500 bp. The 16S rRNA universal probes and primers were designed from the available alignment data [5]. All the in silico analysis of the primers and probes were carried out using Lasergene, version 8.1 software (DNASTAR, Madison, USA). The specificity of the probes and primers were checked thoroughly on all available sequences in the database using NCBI BLAST programme (http://blast.ncbi.nlm.nih.gov/blast.cgi). All the primers and probes were synthesized from Sigma-Aldrich. The details of the probes and primers used are given in Table 2.

Table 1 Organisms used for the development of mPCR/RLB assay

Organism	Isolate name	Source
B. anthracis	BA-1 to BA-18 and BA-21 to BA-29	Human isolates
B. anthracis	WB and O2	Soil isolates
B. anthracis	Sterne	Vaccine strain
Y. pestis	Yp4, Yp8, Yp9, Yp101 to Yp108, YpS1 to YpS3	Human isolates
Y. pestis	Yp111 to Yp117	Rodent isolates
B. melitensis	Bm1 to Bm7	Human isolates
B. melitensis	16 M	Vaccine strain
B. pseudomallei	Bps 2 to Bps 12	Human isolates
B. pseudomallei	Bps Soil	Soil isolate
B. cereus	ATCC 10876, ATCC 10371, ATCC 13061, ATCC 11778	
B. thuringiensis	MTCC 869	
Y. enterocolitica	ATCC 23715, MTCC 3101, MTCC 3100	
Y. pseudotuberculosis	Strain 1b	
Y. kristensenii	ATCC 33639	
Y. federicksenii		
B. abortus	U38, U39	Cattle isolate
B. abortus	NCTC 10093	
B. abortus	S19	Vaccine strain
B. mallei	NCTC 10230	
Salmonella typhimurium	MTCC98	
Salmonella typhi	MTCC734	
Shigella boydi	ATCC9207	
Escherichia coli	*BL-21*	
Leptospira interogans	ATCC23470	

ATCC American type culture collection, *MTCC* microbial type culture collection, *NCTC* national culture type collection

Specificity and Sensitivity Analysis

Single colonies were picked up from brain heart infusion agar plate and resuspended in 100 µl of 10 mM TE buffer (pH 8.0) for DNA extraction. The extracted DNA was tested by PCR with all the primers given in Table 2. The sensitivity analysis was performed based on both DNA dilution and colony forming units of the organisms. For determining DNA detection limit, total genomic DNA concentration was determined and ten fold serial dilution was prepared. The diluted DNA was used directly for the assay. In order to determine the sensitivity, based on colony forming units, cfu was determined from cultures of *B. anthracis*, *Y. pestis*, *B. melitensis* and *B. pseudomallei* and was spiked into human blood of healthy volunteers at concentration starting from 10^5 to 10^1 cfu/ml.

DNA Extraction and Multiplex PCR

DNA was extracted either from 100 µl of spiked blood or colonies of each organism using DNeasy Blood and Tissue kit (Qiagen, GmbH) as per the manufacturer's instructions. Approximately, 5 µl of each DNA was used as a template for the assay. The remaining PCR reaction mixture consisted of 0.2 µM each of primers, 3 mM $MgCl_2$, 200 µM each of dNTP, 20 mM Tris–HCl (pH 8.3), 20 mM KCl, 50 mM $(NH_4)_2$ SO_4 and 1 U of True Start *Taq* polymerase (Fermentas, Lithuana) in a total volume of 25 µl. The mPCR was run with the following conditions: Initial denaturation at 95 °C for 3 min; 40 cycles of 95 °C for 45 s, 60 °C for 90 s and 72 °C for 45 s; final extension for 10 min at 72 °C. From this reaction, 2 µl of the product was run on 1.5 % agarose gel prepared in TBE buffer and the remaining was diluted up to 150 µl with 2 × SSPE/0.1 % SDS for reverse line blot.

Table 2 Oligonucleotide probes and primers used for the study

Probe/primer	Target gene	Organism	5′–3′ Sequence	Genbank accession no.
16S1F[a]	16S rRNA	Most eubacteria	AGAGTTTGATCCTGGCTCAG	
16S1R[a]			CGTATTACCGCGGCTGCTGGCAC	
16S2F[a]	16S rRNA	Most eubacteria	ATTGACGGGGGCCCGCACAAG	
16S2R[a]			TGACGTCATCCCCACCTTCCT	
BaChF[a]	dhp61.183	*B. anthracis*	TGCAATCGATGAGCTAATGAACAAT	NC003997
BaChR[a]			CAGGATGGGTCTCGATTTTGTG	
BaPaF[a]	*pag*	*B. anthracis*	TTCGAAAAGGTTACAGGACGGATT	AF268967
BaPaR[a]			GCTTTAATTGTCGCGAGTGTTTG	
BaLfF[a]	Lethal factor	*B. anthracis*	TCCCGTCTTTATCCCCCTTGT	M29081
BaLfR[a]			ACGGGTTCATATCCTTCTTTTGCAT	
BaEfF[a]	Edema Factor	*B. anthracis*	TCCGTTTGCATCCCGTTTTGTAT	M24074
BaEfR[a]			TCTCCTTTCAGCACATCAATCCTAT	
BaCapF[a]	*capA*	*B. anthracis*	CAGGAGCTATTGCAACGAAAGAAC	NC003981
BaCapR[a]			GGTTTTGGTGATCCCTCTTGAATATT	
YpesChF[a]	*YihN*	*Y. pestis*	CCAAGTAATGACGCCAACCAATATC	NC003143
YpesChR[a]			CTATGCAACAAGACTGGGGGCTAAC	
YpesCafF[a]	*caf1*	*Y. pestis*	CGTTATCGCCATTGCATTATTTG	AL117211
YpesCafR[a]			ACGGTTACGGTTACAGCATCAGTG	
YpesPlaF[a]	*pla*	*Y. pestis*	GGCAACCATTATAACTATTCTGTCCG	AL109969
YpesPlaR[a]			GAACCACCTGTAGCTGTCCAACTG	
YpesLcrF[a]	*lcrV*	*Y. pestis*	ATCGCCGAATACACAATGGGAAT	AL117189
YpesLcrR[a]			CCCCGGTTCTTTTATTCTCACTTCC	
BcspF[a]	BCSP31	*Brucella* spp.	TGCGGGAAGAGGACTGGTATTATGA	M20404
BcspR[a]			GGGTAAAGCGTCGCCAGAAGG	
BmelF[a]	IS117	*Brucella* spp.	TGCCGATCACTTAAGGGCCTTCAT	AE008917
BmelR[a]	BMEI1162	*B. melitensis*	TCGGCTCAGAATAATCCACAGAAGG	AE008917
BpsF[a]	*Orf11*	*B. pseudomallei*	GCCTGCACCGGATTCTCGATA	AF074878
BpsR[a]			ACGTGCATCGGTTTCCAGTGT	
16SUniv1[b]	16S rRNA	Most eubacteria	GACTCCTACGGGAGGCAGCAG	
16SUniv2[b]	16S rRNA	Most eubacteria	TTAAGTCCCGCAACGAGCGCAACCCT	
16SBac[b]	16S rRNA	*Bacillus* sp.	AAGTGCTAGTTGAATAAGCTGGCAC	
16SYer[b]	16S rRNA	*Y.* Sp.	AGCGGGAAGTAGTTTACTACTTTG	
16SBru[b]	16S rRNA	*Brucella* sp.	GTCGCGGTTAGTGGAGACA	
16SBps[b]	16S rRNA	*Burkholderia* sp.	ATCATTCTGGCTAATACCCGGAGT	
BaCh[b]	dhp61.183	*B. anthracis*	CCCACAGCGGCTAAAGAGACCAGTA	
BaPA[b]	*pag*	*B. anthracis*	ACCCCCTTGTGGCAGCTTATCCG	

Table 2 continued

Probe/primer	Target gene	Organism	5′–3′ Sequence	Genbank accession no.
BaLF[b]	Lethal factor	*B. anthracis*	AGGGGAGGAAGCTGTTAAAAAAGAGGCA	
BaEF[b]	Edema factor	*B. anthracis*	AATTTCAGCATGCGTTTTCTTTAGCG	
BaCapA[b]	*capA*	*B. anthracis*	ACGCACTGGGGGAAGAATACGA	
YpCh[b]	*YihN*	*Y. pestis*	GGCCCACACCGATCAACCCTACG	
YpCaf1[b]	*caf1*	*Y. pestis*	CGGGCAGCCAGGATTTCTTTGT	
YpPla[b]	*pla*	*Y. pestis*	AGGGGGTGGACGTCTCTGGCTTC	
YpLcr[b]	*lcrV*	*Y. pestis*	TCAGGTGGATGGGAGCGAGAAAAA	
BmBCSP[b]	BCSP31	*Brucella* sp.	TTGCACAGGCCCCGACATTTTTCCGTATC	
Bmelitensis[b]	BME11162	*B. melitensis*	GCAGGGCGAAGATTGGCGGGTAAGCT	
BpsOrf11[b]	*Orf11*	*B. pseudomallei*	CGCCCAGCAGCAACCCCTCCTCCAG	

F forward, *R* reverse

[a] Primers

[b] Probes

Reverse Line Blot Hybridization

Reverse line blot hybridization was carried out as per the standard protocol of Kong and Gilbert [6]. Briefly, 150 µl of diluted 5′ amino modified oligonucleotide probes at a final concentration of 1–20 pmol/µl were bound to EDAC activated Biodyne C membrane (Pall Gelman Laboratory, USA). After removing the excess oligonucleotide probes, the membrane was deactivated by incubating in 100 mM NaOH for 8 min in a rolling bottle. The membrane was then washed once with 250 ml 2 × SSPE/0.1 % SDS for 5 min at 60 °C. PCR products were diluted to 150 µl volume with 2 × SSPE/0.1 % SDS and denatured at 99 °C in a thermal cycler for 10 min. The denatured were loaded on to their respective lanes and allowed to hybridize with the membrane bound probes. Hybridization was carried out at 52 °C while a more stringent washing step at 60 °C removed any nonspecific binding. Signals were obtained with the help of streptavidin–horseradish peroxidase conjugate (Roche Diagnostics GmBH, Germany) and exposing the membrane to X-Ray film (Kodak, USA) for 5–10 min. All the assembly for probe and sample loading including the hybridization steps were carried out using a 45 lane Miniblotter (Hoefer Inc, CA, USA).

Results

Specificity of the Probes and Primers

Fourteen pairs of primers and 18 probes which did not have any significant hits with other targets in the database were chosen for our assay. The specificity of the primers was first checked by PCR individually prior to hybridization. The primer pairs amplified only the corresponding gene from the specific organisms and did not show any non specific amplification other than the intended targets.

Multiplex PCR

The multiplex PCR amplicons consisted of fragments with size ranging from 280 to 540 bp while in non related organisms only the two internal control bands, corresponding to the 16S rRNA gene, were amplified (Fig. 1). Due to only slight difference in the size of the PCR amplicon, some of the bands overlapped and cannot be differentiated well enough on agarose gel.

Reverse Line Blot Hybridization

The hybridization temperature was kept low at 52 °C for proper hybridization of the amplified DNA to the probes. However, the washing step was carried out at 60 °C in

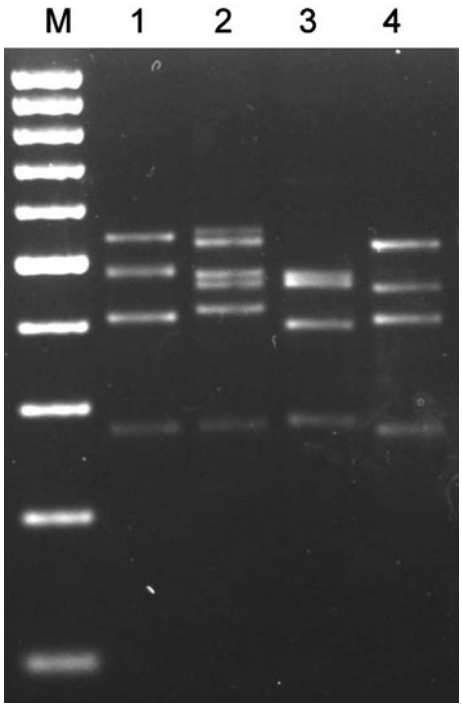

Fig. 1 Agarose gel showing multiplex PCR products from the four organisms individually *Lane M*: 100 bp DNA ladder; *Lane 1*: Multiplex PCR with *B. anthracis* DNA; *Lane 2*: Multiplex PCR with *Y. pestis* DNA; *Lane 3*: Multiplex PCR with *B. melitensis* DNA; *Lane 4*: Multiplex PCR with *B. pseudomallei* DNA

order to eliminate any non specific hybridization. Hybridization assay with purified DNA from bacterial isolates did not show any cross reaction other than their respective target probes. The assay could differentiate the organism distinctively from their closely related species as shown in the Fig. 2. All the other non related but common pathogens which were used for this study did not react with any of the probes except with the 16S rRNA universal control probes.

Sensitivity of the Assay

The sensitivity of the assay was assessed both with purified DNA and with bacteria spiked blood samples. When purified DNA from picked colonies was used, the minimum concentration of detection limit ranged from 0.3 pg to 0.3 ng for different targets. With spiked blood, the detection level was 5×10^2 cfu/ml for *B. anthracis*, 8×10^2 cfu/ml for *Yersinia* species, 1.4×10^2 cfu/ml for *B. melitensis* and 4×10^2 cfu/ml for *B. pseudomallei*.

Discussion

The aim of the current study was to develop a simple, robust and rapid diagnostic platform capable of simultaneous detection and characterization of four BW classified pathogens. A total of 29 isolates of *B. anthracis*, 22 isolates of *Y. pestis*, seven isolates of *B. melitensis* and 13 isolates of *B. pseudomallei* along with their closely related species were included in the study. Since the assay has been designed with the intention of simultaneous detection of genetically unrelated organism, it was not possible to locate a universal gene where upon a single pair of primers can be used to differentiate the pathogen to the species level. Although, 16S rRNA subunit gene has been used for broad classification of organisms, discrimination up to the species level is not possible as the selected organisms for the present work share a very close genetic make up with other members of their respective genus. Firstly, we have used 16S rRNA probes to classify the pathogens based on genera. Our 16S rRNA probes are meant to hybridize the corresponding gene, at least from the pathogenic species within the genus, if not all. We have designed two pairs of universal primers, 16S1F and 16S1R along with 16S2F and 16S2R to amplify two different regions on 16S rRNA gene of most eubacteria. The organism specific 16S rRNA probes lie within these regions. Since the assay is based on two step method, we consider it essential to include a control hybridization probe to eliminate any false negative cases. Two universal probes, 16S Univ1 and 16S Univ2, were designed from the region encompassed by the amplified fragment and served as an internal control (IC) for the assay. For *Brucella*, in addition to the 16S rRNA gene, we have included a probe from BCSP 31 gene which is considered genus specific for all *Brucella* species [7]. These probes and primers proved very specific in identification of organisms to the genus level.

In order to specifically differentiate the pathogen from its closely related species, we have targeted chromosomal gene sequences specific for each pathogen. For *B. anthracis*, we have chosen a unique genomic locus, dhp61.183 [8] and for *Y. pestis*, a chromosomal gene target, *YihN* [9]. *B. melitensis* specific sequence was designed from BMEI1162 gene [10]. The probe and primer for this gene, targets the unique insertion of IS*711* element in the *B. melitensis* genome. The *B. pseudomallei* probe has been designed from a gene coding for its type III secretion system [11]. Plasmid based virulence factors have usually been targeted for both immunological and PCR based detection assays. But recent discovery of virulence plasmids cured *B. anthracis* isolates [12], *B. cereus* harbouring anthrax virulence plasmids [13] and atypical strains of *Y. pestis* lacking virulence plasmids [14] has made the inclusion of a specific chromosomal sequence in the assay vital. Detection of virulence factors along with a species specific chromosomal marker will help to pinpoint the exact identity of the pathogen. This is especially important from biodefence perspective where the use of genetically altered organisms cannot be ruled out. In the case of *B. melitensis* and *B. pseudomallei*, all the targets chosen in

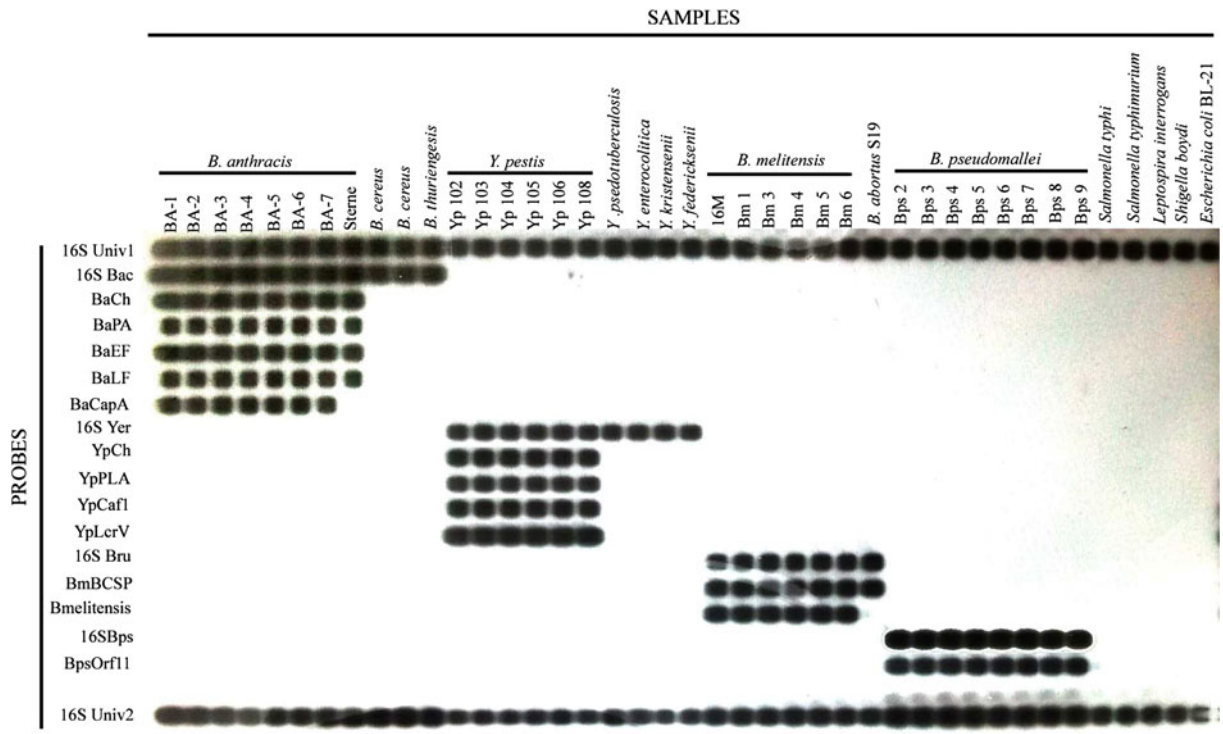

Fig. 2 Reverse line blot with representative organisms for each species Probes 16S Univ 1 and 16S Univ 2 are two universal control probes which are suppose to hybridize all Eubacter 16S rRNA. Probe 16S Bac is *Bacillus* genus specific while BaCh, BaPA, BaEF, BaLF, and BaCapA are *B. anthracis* specific probes. Sterne strain of *B. anthracis* does not show positive signal for BaCapA since it is plasmid pXO2 negative. For *Y. pestis*, 16S Yer is a genus specific probe while YpCh, YpPLA, YpCaf1 and YpLcrV are species specific probes. For *B. melitensis*, 16S Bru and BmBCSP are genus specific probes while *B. melitensis* is a species specific probe. For *B. pseudomallei*, 16S Bps is a genus specific probe while BpsOrf11 is a species specific probe. *Salmonella, Leptospira, Shigella* and *E. coli* samples were included as an unrelated but common pathogen negative control

this study are chromosomal sequences. Our probes based on these gene sequences were very specific and clearly differentiated the species of the organism within the genus.

Apart from these chromosomal targets, the virulence factor based probes includes *capA* (Capsular antigen), *pag* (Protective antigen), *lef* (lethal factor) and *cya* (Edema factor) for *B. anthracis*. These gene sequences originate from two mega plasmids, the pXO1 and pXO2. The *pag*, *lef* and *cya* genes, located in the pXO1 plasmid, are components of a tripartite anthrax toxin complex and together in binary combination produce the lethal toxin and edema toxin. The *capA* gene, which codes for poly-D-glutamic acid capsule is located on the plasmid pXO2 and plays a role in the pathogen evasion of host immune response. The absence of either of the two plasmids renders the organism avirulent [15]. We have included *capA* gene to differentiate the virulent isolates of from avirulent vaccine strains of *B. anthracis*. The virulence factors of *Y. pestis* includes *caf1* (Fraction 1 antigen), *pla* (Plasminogen activator) and *lcrV* (V antigen) which are located on plasmids pMT1, pCP1 and pCD1 respectively. These virulence factors' probes and primers also did not show any cross hybridization except for *Y. pestis lcrV* probe which reacted lightly with *Y. pseudotuberculosis*. However, this cross reaction was eliminated by increasing the level of stringency of the assay. This cross reaction is due to the presence of a homologue of the pCD1 plasmid, a type III secretion system encoding plasmid, in *Y. enterocolitica* and *Y. pseudotuberculosis* [16]. The plasmids, pMT1 and pPCP1, however, exists only in *Y. pestis* and the detection of these targets will confirm the identity of the species involved. The reason for the inclusion of the *lcrV* gene in our assay, in spite of the *lcrV* gene cross reacting with *Y. pseudotuberculosis* even in our BLAST analysis (95 %), is to complement the characterization of the virulence factors of *Y. pestis* in the assay, and not for differentiation of the species. Given the intra-genus similarity in the genetic make up of the pathogens included in this study, none of the probes and primers cross reacted with their closely related organisms except for *lcrV* gene mentioned above. The assay also did not show cross reactivity with any of the commonly encountered clinical pathogens tested in the study either.

In conclusion, the mPCR/RLB assay demonstrated capable of simultaneous detection and thorough characterization of four selected BW agents. The assay has

proven excellent, both in terms of specificity and sensitivity. Although, the present work includes only four BW categorized agents, it can be improved to cover more organisms and targets. It can serve as an important diagnostic platform in BW attack scenario where the threat can include a diversity of organisms and the disease-specific symptoms are not expected to be seen initially. In critical situation where rapid identification of pathogen is required, the assay will have a crucial role in preventing further spread by timely administration of specific therapy and control measures as it can easily be carried out even in small laboratories and health subcentres.

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