

A Novel Semiquantitative Fluorescence-Based Multiplex Polymerase Chain Reaction Assay for Rapid Simultaneous Detection of Bacterial and Parasitic Pathogens from Blood

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A multiplex polymerase chain reaction assay was developed for the rapid simultaneous detection of category A select bacterial agents (*Bacillus anthracis* and *Yersinia pestis*) and parasitic pathogens (*Leishmania* species) in blood using the Cepheid Smart Cycler platform. *B. anthracis* (Sterne) and *Yersinia pseudotuberculosis* were used in the assay for optimization for *B. anthracis* and *Y. pestis*, respectively. The specificity of the target amplicons [protective antigen gene of *B. anthracis* and rRNA genes of other pathogens or human (internal control)] was evaluated by staining the amplicons with SYBR Green I and determining their individual melting temperatures (T_m). As a novel approach for pathogen semiquantitation, the T_m peak height of the amplicon was correlated with a known standard curve of pathogen-spiked samples. This assay was able to detect DNA in blood spiked with less than 50 target cells/ml for all of the pathogens. The sensitivity of this assay in blood was 100% for the detection of *Leishmania donovani* from leishmaniasis patients and *B. anthracis* (Sterne) from symptomatic mice. The time necessary for performing this assay including sample preparation was less than 1.5 hours, making this a potentially useful method for rapidly diagnosing and monitoring the efficacy of drugs or vaccines in infected individuals. (*J Mol Diagn* 2005, 7:268–275)

Blood transfusion saves millions of lives each year. Eighty percent of the world's population lives in developing countries with an access to only 20% of the world's safe blood supply (World Health Organization, press releases,

2000, <http://www.who.int/inf-pr-2000/en/state2000-09.html>). Blood has the potential to be contaminated by numerous pathogens.¹ Contamination of blood or blood products could be accidental, mainly due to donation from infected asymptomatic individuals or a deliberate bioterror attempt to spread the infectious agents. The organisms with maximum potential to be used as biothreats are usually highly virulent and often difficult to diagnose.

Several bacterial and viral pathogens are listed by the Centers for Disease Control and Prevention as category A agents, which may appear in blood during infection. Some of them are gram-positive spore-forming bacteria, *Bacillus anthracis* and *Clostridium botulinum* that cause anthrax and botulism, respectively,² gram-negative bacterium *Yersinia pestis* that causes plague,³ and viruses responsible for various hemorrhagic fevers.^{4,5} Death due to anthrax occurs when the bacteremia reaches 10^7 to 10^8 bacilli/ml of blood.⁶ *Y. enterocolitica*, a common contaminant of packed red blood cells, was responsible for 50% of all clinical sepsis episodes associated with the transfusion of red blood cells, of which 61% were fatal.⁷ Packed red blood cells or whole blood, usually stored at 4°C, can allow the growth of these gram-negative bacteria.⁸ Among the bacteria mentioned above, *B. anthracis* and *Y. pestis* disseminate easily in the environment and result in high mortality rates.^{9,10} Concern about the usage of category A select organisms as bioweapons has increased because of recent intentional dissemination of *B. anthracis* spores in the United States.¹¹ Although the average incubation period for such bacteria in the body before the onset of symptoms is only a few days (2 to 10 days),² asymptomatic individuals could potentially donate infected blood in this window period.

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There are several protozoan parasites that are blood borne, each of which causes a unique severe disease and that are of concern to the blood supply. For example, parasites belonging to the trypanosomatid family such as *Leishmania donovani* and *Leishmania major*, cause the fatal visceral (kala-azar) and cutaneous leishmaniasis, respectively.¹² Similarly, *Trypanosoma brucei* and *Trypanosoma cruzi* are the causative agents for African sleeping sickness and Chagas disease, respectively.¹³ Thousands of immigrants to the United States from Chagas disease endemic regions, carrying asymptomatic chronic *T. cruzi* infections, represent a reservoir population for potential transfusion transmission of *T. cruzi*.¹⁴ At least six *T. cruzi* transfusion-transmitted cases have been reported so far in the United States and Canada.¹⁴ Leishmaniasis currently threatens 1.5 to 2.0 million people annually with an estimated death toll of 50,000 persons/year in 88 countries around the world.¹² The intracellular parasite *Leishmania* is present in blood for an undefined period of time without showing any clinical symptoms during the initial phase of the disease.¹⁵ Such individuals can potentially donate infected blood, because *Leishmania* not only survives blood-banking storage conditions, but also retains its infectivity as demonstrated by studies in animal models, such as hamsters and dogs.^{16,17} *Leishmania* species such as *L. donovani* causing visceral disease have been shown to be transmitted by blood transfusion in several human cases.¹⁵ US army personnel stationed in endemic areas are exposed to *Leishmania* infection. In a recent Centers for Disease Control and Prevention/Department of Defense Morbidity and Mortality Weekly Report, ~500 cases of cutaneous leishmaniasis and 2 visceral cases have been reported from US soldiers deployed in both Iraq and Afghanistan.¹⁸ Hence the Department of Defense and the American Association of Blood Banks implemented a 1-year deferral period for soldiers returning from Iraq after deployment and permanent deferral for a diagnosed case of leishmaniasis. This deferral does not extend to US soldiers in Afghanistan since they have been deferred because of Malaria. The Food and Drug Administration in the United States is in agreement with such a deferral policy as a measure to ensure the safety of the nation's blood supply from transmission of *Leishmania* (Blood Products Advisory Committee Meeting, December, 2003).

Under the circumstances described above rapid and accurate detection of contamination or diagnosis of infection due to either bacterial or parasitic agents in blood is needed. Various methodologies have been reported to diagnose *B. anthracis*, *Y. pestis*, and *Leishmania* species with a wide range of specificity and sensitivity, mostly for an individual organism.^{3,13,19–26} The present study describes the development and evaluation of a single-tube rapid multiplex polymerase chain reaction (PCR) for the simultaneous detection of *B. anthracis*, *Y. pestis*, and Trypanosomatida in blood. In the current study the sensitivity and specificity of a multiplex PCR assay for the detection of the three types of blood borne pathogens are assessed.

Materials and Methods

Bacterial and Parasitic Species and Culture Conditions

Vaccine strain of *B. anthracis* (Sterne 34F2) (Colorado Serum Co., Denver, CO) was cultured in SG sporulation medium.²⁷ *Y. pseudotuberculosis* (Pfeiffer) Smith and Thal [American Type Culture Collection (ATCC) no. 6905] and *Y. enterocolitica* strain 8081 were cultured in Luria-Bertani liquid medium.²⁸ The bacterial cells were stored at -80°C according to standard procedure.²⁸ To calculate cell concentration of the stored cultures, an aliquot of cultures was used to count cells by serial dilution and plating on Luria-Bertani agar plates. Different species of *Leishmania* and *T. brucei* from our laboratory stocks were cultured using published protocols^{29,30} and cells were counted using a Coulter particle counter (Beckman Coulter, Miami, FL). *In vitro* grown *L. donovani* axenic amastigote parasites (strain 1S, clone 2D, World Health Organization designation MHOM/SD/62/1S-CL2D)³¹ that closely resemble the amastigote stage of *Leishmania* found in human macrophages were used to spike *Leishmania* into blood samples.¹²

Target Genes and Design of Primers

The amplicons that were chosen are from the genes that have multiple copies in both selected pathogens and humans. The *B. anthracis* detection target is in the protective antigen gene located on plasmid pXO1, present also in the vaccine strain. The *Y. pestis* target is in the 16S rRNA gene region conserved in all of the *Yersinia* species. Similarly, to detect *Leishmania* species the target is in the conserved region of the 18S rRNA gene. To increase the confidence of the assay and to recognize the false-negatives that may arise because of a reaction failure, an internal control was used. The internal control target is in the human 18S rRNA gene.

To establish a multiplex assay, we identified primer sets specific for these pathogen targets and the human ribosomal RNA control that have similar annealing temperatures and an optimal Mg^{2+} concentration using the Primer 3' program.³² The length of all of the primers was between 18 to 23 bases with an optimum (50%) GC content. Optimum melting temperature (T_m) of the oligos was 58°C at a salt concentration of 50 mmol/L. The primers (sequences not provided for security reasons) of *B. anthracis*, *Yersinia*, and *Leishmania* were screened against the human RepBase library [a database of repetitive DNA sequence elements found in a variety of eukaryotic organisms (<http://www.hgmp.mrc.ac.uk/Software/EMBOSS/Apps/epimer3.html>)] to avoid repetitive regions. The specificity of the primers and avoidance of cross-hybridization with human sequences was assured by testing in BLAST searches (<http://www.ncbi.nlm.nih.gov/blast>). To avoid formation of primer homodimers and heterodimers, the primer sequences were tested in the Amplify 1.2' program (<http://engels.genetics.wisc.edu/amplify>). To discriminate the amplified products, care was taken while

designing the oligos to select different sized amplicons that have different melting temperature values. The lengths of the four amplicons were 99 bp for *B. anthracis*, 121 bp for *Yersinia*, 163 bp *Leishmania*, and 141 bp for the internal control. The amplicon sequences were tested in the Mfold program to avoid secondary structures on DNA where the primers anneal.³³

DNA Extraction from Bacteria, Parasite, and Whole Blood

Total DNA from cultured *B. anthracis* (Sterne), *Y. pseudotuberculosis*, *Leishmania* species, and *T. brucei* was extracted, purified, and measured using the Genome DNA kit (BIO 101, Carlsbad, CA) and manufacturer's protocol. To determine the limit of detection, 200 μ l of healthy anonymous donor human whole blood (National Institutes of Health blood bank) containing heparin as anti-coagulant was seeded with serial dilution of each pathogen and the DNA was extracted with the QIAamp DNA blood mini kit (Qiagen, Valencia, CA). To extract DNA from the blood of visceral leishmaniasis patients, 200 μ l of blood was directly used for the extraction. However, to extract DNA from the mice infected with *B. anthracis* (Sterne) 10 μ l of the blood was mixed with 190 μ l of healthy human blood and used for extraction. The total processing time for a DNA extraction was less than 30 minutes. The DNA at the final step was dissolved in 50 μ l of Qiagen elution buffer and 5 μ l of this was used in each multiplex PCR reaction.

Multiplex PCR Assay for Detection and Semiquantitation of Pathogen Cells

A master mix was prepared (for each 25- μ l reaction volume) containing 1 \times buffer (TaKaRa), 6 mmol/L MgCl₂, 0.25 U Ex Taq (TaKaRa), 0.25 mmol/L dNTP, 0.3 μ mol/L each primer, 1 \times SYBR Green I (vendor stock 10,000 \times ; Molecular Probes, Eugene, OR), and water to 20 μ l and aliquoted into Smart Cycler PCR reaction tubes (Cepheid, Sunnyvale, CA). Then 5 μ l of template DNA was added to each tube. Cycling parameters were: preheat at 94°C for 150 seconds and then 45 two-step cycles of 94°C for 15 seconds and 64°C for 30 seconds. After the last amplification cycle, PCR products were analyzed by melting curve analysis in the Smart Cycler by slowly increasing the temperature to 95°C. Total reaction and melting curve analysis time for a sample was 55 minutes. The reactions were run in triplicate with appropriate controls. The fidelity of the amplified products of all of the organisms was confirmed by individually cloning the amplified products into pCRII-TOPO plasmid (Invitrogen, Carlsbad, CA) and sequencing.

The lowest level of detection of each pathogen was determined by using the extracted DNA obtained from 200 μ l of blood spiked with serial dilutions of cells from each individual pathogen (ie, 10,000, 1000, 100, 10, and 5 cells). To calculate the pathogen load in the blood, a standard curve based on the fluorescence peak height of

the amplified products and pathogen cell number in blood was generated.

Detection of Pathogens from Blood Samples of *B. anthracis*-Infected Mice and Leishmaniasis Patients

The sensitivity of the multiplex assay was tested on the blood obtained from mice infected with *B. anthracis* (Sterne). The mice were inoculated intraperitoneally with a target dose (lethal dose 90%) of 2 to 2.5 \times 10⁷ spores of bacteria essentially as described previously.³⁴ Blood samples were collected in the presence of anti-coagulant from these infected mice at different time points after challenge. Samples from noninoculated mice were included in the assay as negative controls.

Blood samples were collected from 11 kala azar (visceral leishmaniasis) patients from Bihar, India, and reporting to Safdarjung Hospital, New Delhi, India, at the pretreatment stage. The patients exhibited characteristic disease symptoms such as fever, hepatosplenomegaly, anemia, and leukopenia. Only patients that were clinically diagnosed for kala azar and found positive for the presence of the parasite in bone marrow aspirates at the pretreatment stage²⁶ were used in this study. In addition, blood samples obtained from three patients after treatment with anti-leishmanial drugs were also included in the study. All of the collected blood samples were heparinized, their identity was blinded and stored at 4°C. The coding of the clinical status of the patient samples were broken after the assay was performed and results were then analyzed. All of the animal and human experiments were done under National Institutes of Health guidelines for animal and human protection (<http://oacu.od.nih.gov/ARAC>).

Results

Evaluation of the Multiplex PCR Assay

Because the virulent strain of *B. anthracis* is a select agent, we used the animal vaccine (Sterne) strain for the assay standardization. Because both *Y. pestis* and *Y. enterocolitica* are infectious, the closely related species *Y. pseudotuberculosis*, which is not a human pathogen was used as a surrogate. *L. donovani* cells were used to test the primer set that has a target in all of the *Leishmania* and *Trypanosoma* species. To test the multiplex assay, the DNA of *B. anthracis* (Sterne) (100 fg), *Y. pseudotuberculosis* (100 fg), and *L. donovani* (50 pg) was mixed with human DNA [a 10th of the DNA (~300 ng) obtained from 200 μ l of blood] and used in a multiplex PCR. Amplification of all of the four amplicons was observed by the appearance of their expected fluorescence T_m peaks (Figure 1A). The T_m peak for *B. anthracis* (Sterne) was 82.2°C, for *Y. pseudotuberculosis* was 87.3°C, for *L. donovani* was 86.1°C, and for the human control was 88.8°C. The agarose gel picture of the PCR reaction shows the amplified products of all of the organisms with expected size DNA fragments (Figure 1B). The fidelity of

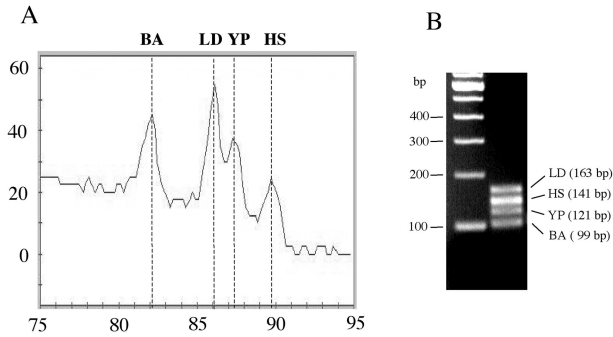


Figure 1. A: Melt derivative analysis of the multiplex fluorescence PCR showing the amplification of specific products of *B. anthracis* (Sterne) (BA) with Tm 82.2°C, *L. donovani* (LD) with Tm 86.1°C, *Y. pseudotuberculosis* (YP) with Tm 87.3°C, and the internal human control (HS) with Tm 88.8°C. **B:** Agarose gel confirmation of the products of the multiplex PCR stained with ethidium bromide from **A** showing the amplified products of: LD, *L. donovani*; HS, human; YP, *Y. pseudotuberculosis*; and BA, *B. anthracis* (Sterne).

the sequence of the amplified products was further confirmed by sequence analysis.

After confirming the feasibility of the multiplex assay conducted using the DNA of the pathogens directly, the lower level of detection (LOD) of the pathogens in the blood was determined by doing the assay on DNA obtained from 200 µl of whole blood spiked with a known number of *B. anthracis* (Sterne) spores, 10,000, 1000, 100, 10, 5, and 0. A Tm peak corresponding to the amplified product of *B. anthracis* (Sterne) was observed at 82°C at all of the concentrations (Figure 2A). We also observed a gradual reduction in the height of the *B. anthracis* Tm peaks (Figure 2A) and the intensity of the

amplified DNA bands of *B. anthracis* on the gel (Figure 2B) with the decreasing number of pathogen spores in the blood. However, in all of the reactions, the height of the internal control (human rRNA gene fragment amplification) Tm peak remained constant. We have used the height of the fluorescence peak above baseline of *B. anthracis* (Sterne) of different spore concentrations in plotting a standard graph for quantification (Figure 2C). All of the negative samples (nine) had baseline fluorescence. Similar studies on the determination of LOD were performed for the other two organisms, *Y. pseudotuberculosis* and *L. donovani*, and shown in Figure 2C. The analysis showed that the assay system can detect <10 cells per 200 µl of blood for all of the three pathogens.

Specificity and Sensitivity of PCR

Each of the primer sets added in the multiplex reaction amplified its product only in the presence of the DNA from its corresponding organism. When the primer sets were tested individually on the templates of other organisms such as *Escherichia coli* and *Bacillus cereus* they did not amplify any DNA fragment (data not included). The primers neither cross-hybridized with each other nor did they react with any amplified products of other pathogens in the assay. They did not amplify any nonspecific fragments other than the four expected amplicons. However, *Yersinia* primers also amplified DNA from *Y. enterocolitica* (with Tm peak at 87°C) when tested in a separate reaction (data not shown). Similarly, the primers designed for *Leishmania* recognized, in addition to *L. donovani*, the

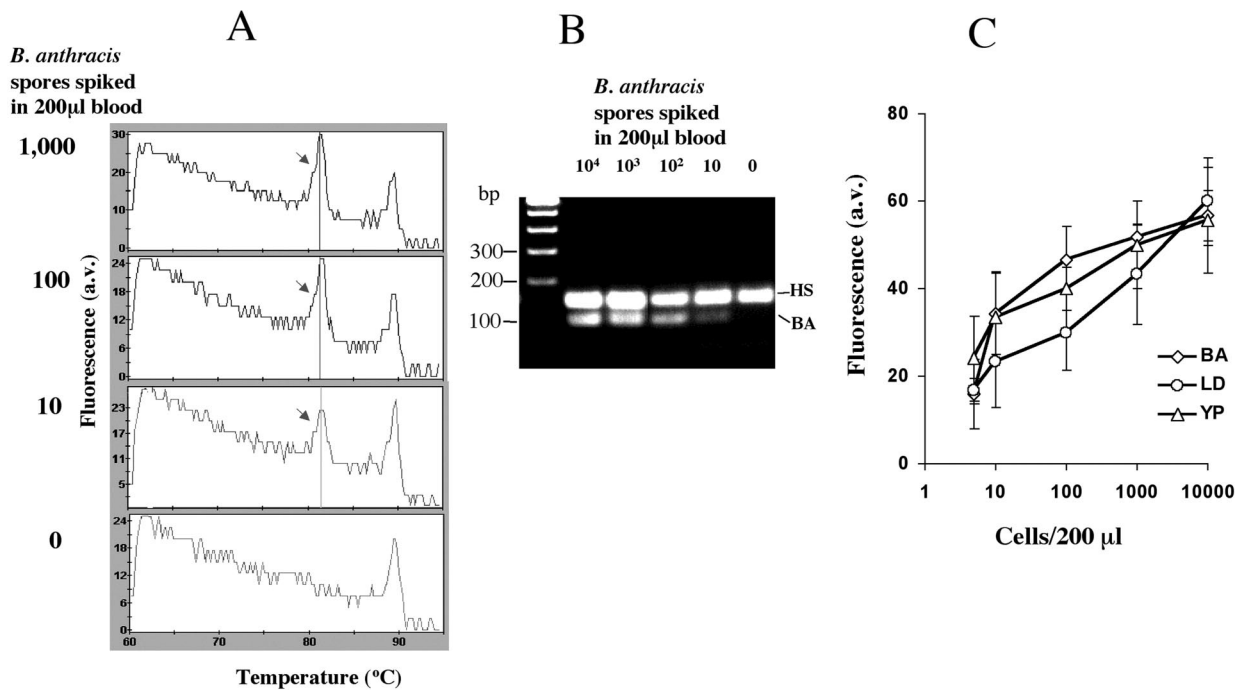


Figure 2. A: Melt derivative analysis of the multiplex PCR assay with human blood carrying a known number of spiked *B. anthracis* (Sterne) spores. **B:** Agarose gel confirmation of the products of the multiplex PCR showing the linear relationship between the logarithm of the number of spores spiked and the amount of amplified band intensity of *B. anthracis* (Sterne). **C:** Quantitation graph showing the linear relationship between the height of the fluorescence peak above baseline of the specific amplicons (y axis) and the logarithm of the number of bacterial or parasitic pathogen cells individually spiked in 200 µl of blood (x axis). BA, *B. anthracis* (Sterne); YP, *Y. pseudotuberculosis*; LD, *L. donovani*; a.v., arbitrary value. The data represent the means ± SD of three independent experiments.

Table 1. Multiplex Fluorescence PCR with Blood Samples from Mice Infected with the Spores of *B. anthracis* (Sterne)

Blood collection time (hours after challenge)	Total number of samples tested	Number of PCR-positive samples (% positives in parentheses)	Approximate <i>B. anthracis</i> (Sterne) cell number in blood per ml
0	6	0	ND
12	6	0	ND
24	4	1 (25)	200 to 1000
36	3	2 (66)	1×10^4 to 10^5
48	2	2 (100)	1×10^4 to 10^5

ND, not detected.

DNA extracted from other closely related *Leishmania* and *Trypanosoma* species that cause different diseases, namely *L. chagasi*, *L. infantum*, and *L. mexicana* that cause visceral leishmaniasis; *L. major* and *L. tropica* that cause cutaneous leishmaniasis; *L. braziliensis* that causes mucocutaneous leishmaniasis; and *Trypanosoma brucei* that causes African sleeping sickness (data not shown).

Detection of Anthrax in Blood Samples from in Vivo-Infected Mice Using Multiplex PCR

Multiplex PCR analysis was conducted with blood samples collected during a time course after infection from laboratory mice inoculated with *B. anthracis* (Sterne). The sample type and PCR results are shown in Table 1. Multiplex PCR conducted on the blood samples at 0 (prebleed) and 12 hours after the intraperitoneal injection of the spores of *B. anthracis* (Sterne) did not show the fluorescence peak for *B. anthracis* (Table 1). However, blood samples collected at 24-, 36-, and 48-hour time points after the inoculation were positive for the bacteria at an increasing rate with time after infection (Table 1). The bacteremia in the animal that tested PCR-positive after 24 hours of exposure was estimated to be 200 to 1000 cells/ml (cell concentration range obtained from the Tm peak standard curve) (Figure 2C). Similarly, four of five animals that were positive after 36 to 48 hours of exposure had bacteremia in their blood between 10^4 to 10^5 cells/ml. All these bacteremic mice were lethargic with scruffy hair and died within 24 hours.

Detection of *L. donovani* in the Blood Samples from Visceral Patients Using Multiplex PCR

The multiplex PCR assay was evaluated with the blood samples collected from 11 leishmaniasis patients who were identified as positive for the presence of the parasite (Table 2). The approximate number of parasites calculated with the help of the standard graph ranged from 50 to 1000 cells per ml of blood in these patients. Whereas samples obtained from three patients who had

undergone drug treatment showed no amplification for the presence of the parasite (Table 2).

Discussion

Individual detection of either *B. anthracis*,^{19,20,24} *Y. pestis*,^{22,23} or *Leishmania*^{26,35} through conventional or real-time PCR has been reported. With an increased concern for blood safety due to emerging pathogens and the threat of bioterror agents, which can be transmissible through blood, the need for rapid pathogen detection becomes essential. The cost and the logistical burden of testing for many phylogenetically diverse pathogens call for a multiplex assay. However, little is known on the simultaneous detection of pathogens of diverse genera through multiplex PCR. Technological hurdles to detection in the unique matrix of whole blood and the increase in complexity of the PCR reaction that occurs when run in a multiplex format have slowed development of such an assay. There is a need to develop tests that could be used for both donor screening and diagnosis ideally based on multiplexing for such pathogens. In the present study, we have developed an assay that can simultaneously identify the presence of both bacterial (*B. anthracis* and *Y. pestis*) and parasitic (Trypanosomatid species) pathogens in blood. We selected target genes that are present in multiple copies in these organisms to achieve enhanced assay sensitivity.

Specific primer sets were designed to detect *B. anthracis*, *Yersinia*, and *Leishmania* to work uniformly in a multiplex PCR reaction using SYBR Green I in a Smart Cycler platform. The primers were generated such that each of the primer sets of an organism amplify a distinct length of amplicon so that the amplified products have distinct melting temperatures and could be resolved on agarose gel if needed. We performed melt-derivative analysis of the PCR products and did not rely on the cycle threshold (CT) value, because the CT value would be a collective fluorescence of all of the amplified products, stained with SYBR Green I in this multiplex reaction. Melt derivatives obtained from the melting curve analysis conducted at

Table 2. Multiplex Fluorescence PCR with Blood Samples from Visceral Leishmaniasis Patients Infected with *L. donovani*

Source of blood samples	Total number of samples tested	Number of PCR-positive samples	Approximate <i>Leishmania</i> cell number in blood per ml
Patients with VL	11	11	50 to 1000
VL patients after treatment	3	0	ND

ND, not detected.

the end of the last PCR cycle resolve the amplified products into individual peaks depending on their T_m values. T_m value differentiates the amplified products based on their length, sequence, and GC content. Such an analysis in this report resolved all of the four amplicons into four distinctive T_m peaks.

We are unaware of any reports with a method to quantify the pathogen cells in biological samples in a multiplex PCR using SYBR Green I-based melting curve analysis. Hence, we have used the T_m peak heights of the products that correspond proportionally to the approximate pathogen cell number per unit volume of blood as a novel way to achieve semiquantitative measure of each pathogen.

Because SYBR Green I can also bind nonspecifically to any amplified DNA, steps were taken to avoid nonspecific amplifications. The enzyme, TaKaRa EX *Taq* polymerase (TaKaRa Shuzo Co., Ltd., Shiga, Japan) premixed with neutralizing monoclonal antibody to the polymerase, that requires hot start for the release of the antibody that activates the polymerase and reduces nonspecific amplification was used in the assay. The reaction samples were hot started at 95°C for 150 seconds before the first denaturation step of thermocycling. A combined PCR annealing and extension of the product was accomplished at a relatively high temperature, ie, 64°C for 30 seconds in each cycle. These features probably are the reason that we did not observe any nonspecific DNA amplification in the reaction. In earlier studies by others in the blood samples spiked with either *B. anthracis* (Sterne),²⁵ *Y. enterocolitica*,³⁶ or *Leishmania infantum*,^{37,38} the lowest limits of detection observed were 400, 8, and 10 to 100 cells/ml, respectively. Whereas in this study, the lower detection level achieved for three pathogens was <50 cells/ml. This suggests that the assay presented here has a higher sensitivity for *B. anthracis* and *Leishmania* detection in blood samples. This assay is rapid and takes less than 1.5 hours to detect any of these organisms either individually or simultaneously if present in blood. There is no such rapid method designed for identifying *Yersinia* sp. in blood. However, an assay developed by Mayo-Roche to detect *B. anthracis* individually in blood takes <1.5 hours.³⁹ Previously tests developed to detect *Leishmania* in blood using fluorescence PCR have shown to take >2 hours.^{40,41}

In addition to the high specificity of the primers selected for all of the three pathogens, primers for *Yersinia* and *Leishmania* could also detect related *Yersinia* and *Leishmania* species. For example, the primers of *Y. pestis* also recognized the DNA of *Y. enterocolitica*, an another blood borne species of *Yersinia* that causes septicemia disorder. Similarly the primers of *Leishmania* recognized DNA of several species of *Leishmania* and *T. brucei* confirming that these primers can identify other blood-borne Trypanosomatid parasites as well. Thus the assay has the ability to detect a limited group of organisms that are all important to detect for blood safety. Primer sets to identify the individual pathogens are available and could be adapted to the Smart Cycler platform. We are in the process of testing the primers described in this study for

virulent forms of *B. anthracis* and *Y. pestis* to ensure their ability to detect the corresponding virulent agents.

The test was also validated successfully using peripheral blood samples obtained from mice infected with *B. anthracis* (Sterne) and from visceral leishmaniasis patients. The sensitivity of *B. anthracis* (Sterne) detection with either regular or real-time PCR using tissue materials other than blood reported earlier was between 89 to 100%.^{21,42} Similarly, reports on detection of *Leishmania* species from blood samples using a PCR assay, have shown 45 to 96% sensitivity with different degree of sample sizes.^{26,43-49} The sensitivity of the current multiplex PCR assay to detect *Leishmania* or *B. anthracis* (Sterne) in blood is 100% from either patients or symptomatic mice, respectively. Infected mice that failed to give a positive PCR test were not symptomatic at the time of collection of the blood sample implying a very low level of bacteremia.

In conclusion, unique primer sets, careful optimization of the conditions, extensive development, and testing with laboratory cultured pathogens spiked into human blood have resulted in an assay that proved capable of detecting *Leishmania* and *B. anthracis* (Sterne) from infected human and mouse blood samples, respectively. This assay meets the requirement of simultaneous, specific, sensitive, and rapid detection of more than one divergent blood-borne pathogen in a single reaction tube. This semiquantitative assay has the potential to be automated and incorporated into high-throughput screening. Optimization toward increasing the number of pathogens detected in a multiplex format and high-throughput screening is under exploration. This assay also has the potential to be used for other body fluid samples. Similar multiplex assays could be designed for other biological agents that pose a bioterror threat.

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