Establish an allele-specific real-time PCR for *Leishmania* species identification

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**Abstract**

**Background:** Leishmaniasis is a serious neglected tropical disease that may lead to life-threatening outcome, which species are closely related to clinical diagnosis and patient management. The current *Leishmania* species determination method is not appropriate for clinical application. New *Leishmania* species identification tool is needed using clinical samples directly without isolation and cultivation of parasites.

**Methods:** A probe-based allele-specific real-time PCR assay was established for *Leishmania* species identification between *Leishmania donovani* and L. *infantum* for visceral leishmaniasis (VL) and among L. *major*, L. *tropica* and L. *donovani/L. infantum* for cutaneous leishmaniasis (CL), targeting hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and spermidine synthase (SPDSYN) gene with their species-specific single nucleotide polymorphisms (SNPs). The limit of detection of this assay was evaluated based on 8 repeated tests with intra-assay standard deviation < 0.5 and inter-assay coefficients of variability < 5%. The specificity of this assay was tested with DNA samples obtained from *Plasmodium falciparum*, *Toxoplasma gondii*, *Brucella melitensis* and *Orientaltsutsugamushi*. Total 42 clinical specimens were used to evaluate the ability of this assay for *Leishmania* species identification. The phylogenetic tree was constructed using HGPRT and SPDSYN gene fragments to validate the performance of this assay.

**Results:** This new method was able to detect 3 and 12 parasites/reaction for VL and CL respectively, and exhibited no cross-reaction with *P. falciparum*, *T. gondii*, *B. melitensis*, *O. tsutsugamushi* and non-target species of *Leishmania*. Twenty-two samples from VL patients were identified as *L. donovani* (*n* = 3) and *L. infantum* (*n* = 19), and 20 specimens from CL patients were identified as *L. major* (*n* = 20), providing an agreement of 100% compared with sequencing results. For further validation, 29 sequences of HGPRT fragment from nine *Leishmania* species and 22 sequences from VL patients were used for phylogenetic analysis, which agreed with the results of this new method. Similar results were obtained with 43 sequences of SPDSYN fragment from 18 *Leishmania* species and 20 sequences from CL patients.

**Conclusions:** Our assay provides a rapid and accurate tool for *Leishmania* species identification which is applicable for species-adapted therapeutic schedule and patient management.

**Keywords:** *Leishmania*, Species identification, Allele-specific real-time PCR, SNPs

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**Background**

Leishmaniasis is a zoonotic disease caused by as many as 21 species of *Leishmania*, which can lead to lethal or traumatic outcome and associated social stigmatization [1]. The vectors and animal hosts of *Leishmania* present diversity and intersectionality, making the diseases more complicated to control. Due to the infection with different species of *Leishmania*, many subclinical infections have no symptoms and many patients exhibit various clinical manifestations [2, 3]. Typically, visceral leishmaniasis (VL) is caused by *L. donovani* and *L. infantum*, which is a serious infection with internal organs and bone marrow and will have fatal consequence without...
treatment in time [4]. Cutaneous leishmaniasis (CL) and mucosal leishmaniasis (ML) are limited to the skin and mucous membranes, and caused by different Leishmania species. CL is caused by L. major, L. tropica and L. infantum which are prevalent around the Mediterranean basin, the Middle East, the horn of Africa, and the Indian subcontinent, and L. amazonensis, L. chagasi (sometimes still referred to as L. infantum in South America), L. mexicana, L. dohotti, L. braziliensis and L. guyanensis which are prevalent around Middle and South America [5]. L. braziliensis and L. aethiopica can cause overt ML [6]. Cured VL, infected with L. infantum and L. donovani, sometimes occurs post kala-azar dermal leishmaniasis (PKDL) [7, 8].

As different Leishmania species exhibit various virulence level, genetic heterogeneity and responses to chemical drugs, the outcome tended to be better when therapy was species-directed performed [9–11]. For instance, L. major, L. donovani, L. braziliensis (in Guatemala) and L. tropica are more sensitive to antimony compared to L. aethiopica, L. panamensis and L. braziliensis (in Brazil). Miltefosine is an effective drug for treating CL caused by L. guyanensis, L. panamensis and L. donovani, whereas CL caused by L. infantum and L. braziliensis exhibit more resistant to it. Unlike L. tropica, L. major, L. mexicana and L. braziliensis are more susceptible for paromomycin (PM). Amphotericin B is recommended to treat CL caused by L. tropica, L. braziliensis, L. major and L. aethiopica but not for L. infantum [12–16]. Further, as there are co-infections with different Leishmania species, it will lead to different pathogenicity and drug sensitivity which make the treatment more complicated [17, 18]. Thus, Leishmania species identification is important in treatment and patient management, including pharmacological selection, appropriate treatment determination (intralesional, intramuscular, oral systemic, or parenteral) and monitoring potential infection sequelae [19–22].

Traditional Leishmania diagnostic techniques, such as microscopic examination, protozoan culture in vitro and serological immunoassay, cannot identify Leishmania species. In present clinical practice, it is still based on empirical judgment according to the information of local epidemiology. However, it could make inappropriate determination for traveler and co-infections with different species [1]. There are some techniques were developed to discriminate Leishmania species, such as sequencing of individual gene, restriction fragment length polymorphism (RFLP), high resolution melting, multilocus sequencing typing and mass spectrometry [21, 23–30]. As the World Health Organization recommended, the “gold standard” method used to identify Leishmania species is multi-site enzyme electrophoresis (MLEE), which requires culture of parasites [2]. However, some Leishmania species are difficult to culture in vitro with cumbersome experiment procedure which also makes the results among different laboratories incomparable. Although some probe based real-time PCR assays were developed for Leishmania species identification, they are mainly focused on L. mexicana, L. braziliensis, L. peruviana and L. major for CL and not suitable for other common clinical infection related species [31, 32]. Thus, for clinical applications, a tool for Leishmania species identification among common clinical pathogens, such as L. donovani, L. infantum, L. major and L. tropica, is needed to be developed using clinical samples directly without isolation and cultivation of parasites.

In this study, to identify Leishmania species, hypoxanthine-guanine phosphoribosyl transferase (HGPRT) and spermidine synthase (SPDSYN) genes were selected from 34 housekeeping genes. Our results showed that, HGPRT gene with species-specific single nucleotide polymorphisms (SNPs) can identify parasite species between L. donovani and L. infantum for VL, and SPDSYN gene with species-specific SNPs can distinguish parasite species among L. major, L. tropica and L. donovani/L. infantum for CL. Thus, an allele-specific real-time PCR technique was established for Leishmania species identification with clinical specimens from VL and CL patients.

Material and methods
 Patients and samples
A total of 42 clinical samples from patients at Beijing Friendship Hospital, Capital Medical University from July 2015 to Sep 2021 (Table 1). The bone marrow (n = 22) and skin lesion tissue (n = 20) were collected from patients with VL and CL individually for allele-specific real-time PCR testing. VL patients presented with symptoms such as fever, splenomegaly and/or hepatomegaly, Leishmania amastigotes found in their bone marrow samples under microscope or PCR positive or Leishmania parasite culture positive. CL patients appeared as ulcer and nodule/plaques features in which Leishmania amastigotes were identified under microscopy. All bone marrow and skin lesion tissue were stored at liquid nitrogen till use. DNA samples of Plasmodium falciparum, Toxoplasma gondii, Brucella melitensis and Orientia tsutsugamushi were used as non-leishmaniasis controls.

Potential target fragment selection
Out of 34 genes of Leishmania with sequence polymorphism previously published, 21 were further analyzed according to the inclusion criteria as follows: first, these gene fragments were shown as markers for the molecular characterization of Leishmania strains and species; second, they are common genetic polymorphism sites for the four species (L. donovani, L. infantum, L. major and
| Diseases | Patient ID | Age | Gender | Diagnosis basis | Symptoms | Anti-rk39 | Etiology | Laboratory test | Imaging | Combined infections | Parasite load | Treatment outcome | Combined HPS |
|----------|------------|-----|--------|----------------|----------|---------|---------|---------------|---------|-------------------|-------------|-------------------|-------------|
|          |            |     |        | Region         |          |         |         |               |         |                   |             |                   |             |
| VL       | 1          | 50  | M      | Yangquan,     | Fever,   | +       | Culture | 3.4           | 3.6     | 11.7             | 44.9        | Enlarged spleen  | HBV, Mycoplasma |
|          |            |     |        | Shanxi, China  | cough,   |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | diarrhea,|         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | abdominal|         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | pain,    |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | ascites  |         |         |               |         |                   |             |                   |             |
|          | 2          | 51  | M      | Yangquan,     | Nausea   | +       | Microscopy| 2.3           | 3.7     | 125              | 59          | Enlarged liver and spleen | Herpes simplex virus, EB virus |
|          |            |     |        | Shanxi, China  | and vomit, |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | appetite,|         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | oily,    |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | weight   |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | loss     |         |         |               |         |                   |             |                   |             |
|          | 3          | 1   | F      | Yangquan,     | Fever,   | +       | Microscopy| 2.6           | 2.7     | 81               | 34.1        | Enlarged liver and spleen | Bacterial pneumonia |
|          |            |     |        | Shanxi, China  | list-     |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | lessness |         |         |               |         |                   |             |                   |             |
|          | 4          | 2   | M      | Yangquan,     | Fever    | +       | PCR [54] | 4.7           | 4.6     | 90               | 48.7        | Enlarged liver and spleen | Mycoplasma, Rickettsia Q fever |
|          |            |     |        | Shanxi, China  |          |         |         |               |         |                   |             |                   |             |
|          | 5          | 35  | F      | Linfen,       | Fever,   | +       | Microscopy| 0.6           | 2.7     | 39               | 27.2        | Enlarged spleen  | Mycoplasma |
|          |            |     |        | Shanxi, China  | chills   |         |         |               |         |                   |             |                   |             |
|          | 6          | 66  | M      | Pingding,     | Fever    | +       | Microscopy| 1.6           | 3.2     | 87               | 24.2        | Enlarged spleen  | None |
|          |            |     |        | Shanxi, China  | cough,   |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | fatigue  |         |         |               |         |                   |             |                   |             |
|          | 7          | 53  | M      | Yangquan,     | Fever,   | +       | Microscopy| 3.6           | 3.2     | 156              | 63.1        | Enlarged liver and spleen | Fungal and bacterial pneumonia |
|          |            |     |        | Shanxi, China  | night    |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | sweats   |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                | chills   |         |         |               |         |                   |             |                   |             |
|          | 8          | 44  | M      | Pingding,     | Fever    | −       | Microscopy| 1.7           | 2.3     | 44               | 29.7        | Enlarged spleen  | Candida albicans |
|          |            |     |        | Shanxi, China  | shortness of breath, fatigue, profuse sweating |         |         |               |         |                   |             |                   |             |
|          | 9          | 1   | F      | Xingtai,      | Fever    | +       | Microscopy| 11.8          | 4.2     | 281              | 38.8        | Enlarged spleen  | Neisseria, Mycoplasma |
|          |            |     |        | Hebei, China   |          |         |         |               |         |                   |             |                   |             |
|          |            |     |        |                |          |         |         |               |         |                   |             |                   |             |
| Diseases | Patient ID | Age | Gender | Region            | Symptoms                          | Anti-rk39 | Etiology          | Laboratory test | Imaging | Combined infections | Parasite load | Treatment outcome | Combined HPS |
|----------|------------|-----|--------|-------------------|-----------------------------------|-----------|------------------|-----------------|---------|---------------------|---------------|-------------------|-------------|
|          | 10         | 33  | M      | Weinan, Shaanxi, China | Fever, cough, expectoration | -         | Microscopy       | WBC 13 RBC 4.5 PLT 68 ALB 22.2 GLB 21.3 | Enlarged spleen | Fungal and bacterial pneumonia, Cytomegalovirus | $1.5 \times 10^7$ | Death          | Yes          |
|          | 11         | 42  | M      | Bayan-naer, Inner Mongolia, China | Fever, chills | +         | Microscopy       | WBC 2.06 RBC 3.18 PLT 453 ALB 31.7 GLB 28.5 | Enlarged spleen | Epstein-Barr virus | $1.0 \times 10^8$ | Under treatment | Yes          |
|          | 12         | 26  | M      | Yangquan, Shanxi, China | Fever, chills | +         | Microscopy       | WBC 2.5 RBC 2.4 PLT 51 ALB 27.6 GLB 41 | Enlarged spleen | None | $1.7 \times 10^7$ | Cure | Yes          |
|          | 13         | 26  | F      | Longnan, Gansu, China | Fever, chills | +         | Microscopy       | WBC 4.5 RBC 2.8 PLT 124 ALB 32.6 GLB 30.8 | Enlarged liver and spleen | None | $1.4 \times 10^6$ | Cure | Yes          |
|          | 14         | 66  | F      | Yangquan, Shanxi, China | Chest tightness, fatigue, cough | +         | Microscopy       | WBC 2.4 RBC 2.8 PLT 64 ALB 25.6 GLB 74.7 | Enlarged liver and spleen | Mycoplasma | $6.3 \times 10^6$ | Cure | No           |
|          | 15         | 32  | M      | Yangquan, Shanxi, China | Fever, chills, fatigue, headache, sweat profusely, cough | +         | Microscopy       | WBC 1.7 RBC 3.0 PLT 91 ALB 35.9 GLB 81.1 | Enlarged spleen | None | None | Under treatment | No           |
|          | 16         | 47  | F      | Yangquan, Shanxi, China | Fatigue, fever, chills, nausea and vomit | +         | Microscopy       | WBC 1 RBC 2.7 PLT 105 ALB 22.4 GLB 83.4 | Enlarged spleen | Mycoplasma, Epstein-Barr virus, Sarkozy virus | $1.7 \times 10^5$ | Under treatment | No           |
|          | 17         | 3   | M      | Yangquan, Shanxi, China | Fever, listlessness, expectoration, vomit, abdominal pain, diarrhea | +         | Microscopy       | WBC 3.9 RBC 4.2 PLT 70 ALB 33.4 GLB 44 | Enlarged spleen | None | $7.9 \times 10^3$ | Under treatment | No           |
| Diseases | Patient ID | Age | Gender | Diagnosis basis | Region | Symptoms | Anti-rk39 | Etiology | Laboratory test | RBC | PLT | ALB | GLB | Combined infections | Parasite load | Treatment outcome | Combined HPS |
|----------|------------|-----|--------|-----------------|--------|----------|----------|----------|-----------------|-----|-----|-----|-----|---------------------|-------------|-------------------|-------------|
| CL       | 18         | 30  | M      | Fever, fatigue  | Yangquan, Shanxi, China | No obvious symptoms | +       | Culture | 7.4         | 3.4 | 268 | 203 | 135 | enlarged spleen | 3.0 × 10^6 | Cure              | No          |
| CL       | 19         | 28  | F      | Fever          | Gansu, China | No obvious symptoms | +       | PCR [54] | 3.5         | 4.2 | 125 | 33.5 | 26.2 | enlarged spleen | un          | Under treatment | No          |
| CL       | 20         | 80  | M      | Cough, fatigue | Shanxi, China | No obvious symptoms | +       | Microscopy | 2.4         | 4.3 | 134 | 27.4 | 66.6 | enlarged spleen | 1.5 × 10^6 | Cure              | No          |
| CL       | 21         | 61  | M      | Fever          | Beijing, China | Fever | +       | Microscopy | 5.8         | 4.7 | 148 | 34.4 | 38.3 | enlarged spleen | None | Under treatment | Yes         |
| CL       | 22         | 52  | F      | No obvious symptoms | Shanxi, China | No obvious symptoms | -       | Microscopy | 1.5         | 4.6 | 191 | 42.8 | 26.8 | enlarged spleen | None         | Under treatment | No          |
| CL       | 23         | 42  | M      | Multiple skin ulcers | Iraq | Multiple skin ulcers | -       | Microscopy | 6           | 5   | 280 | 41.6 | 31.7 | Normal         | 1.7 × 10^7 | Cure              | No          |
| CL       | 24         | 47  | M      | Multiple skin ulcers | Iraq | Multiple skin ulcers | -       | Culture    | 5.5         | 4.5 | 182 | 35.8 | 31.1 | Normal         | 1.5 × 10^8 | Cure              |             |
| CL       | 25         | 35  | M      | Multiple skin ulcers | Iraq | Multiple skin ulcers | -       | Culture    | 5.6         | 4.6 | 232 | 41.4 | 28  | Normal         | 5.4 × 10^7 | Cure              |             |
| CL       | 26         | 55  | F      | Multiple skin ulcers | Morocco | Multiple skin ulcers | +       | Microscopy | 5.2         | 4.4 | 196 | 38.4 | 31.8 | Normal         | 1.0 × 10^8 | Cure              |             |
| CL       | 27         | 48  | M      | Multiple skin ulcers | Iraq | Multiple skin ulcers | -       | Microscopy | 3.8         | 4.1 | 164 | 35.9 | 23.4 | Normal         | 4.9 × 10^7 | Cure              |             |
| CL       | 28         | 29  | M      | Multiple skin ulcers | Iraq | Multiple skin ulcers | -       | Microscopy | 4.9         | 5.2 | 264 | 40.8 | 22.7 | Normal         | 1.4 × 10^6 | Cure              |             |
| CL       | 29         | 40  | M      | Multiple skin ulcers | Iraq | Multiple skin ulcers | +       | Microscopy | 7.7         | 4.94 | 202 | 40.2 | 26.4 | Normal         | 8.0 × 10^4 | Cure              |             |
| CL       | 30         | 34  | M      | Multiple skin ulcers | Iraq | Multiple skin ulcers | -       | Microscopy | 3.9         | 4.41 | 161 | 38.3 | 24.2 | Normal         | 1.8 × 10^8 | Cure              |             |
| CL       | 31         | 43  | M      | Multiple skin ulcers | Iraq | Multiple skin ulcers | -       | Microscopy | 4.5         | 4.6 | 207 | 39.5 | 29.9 | Normal         | 9.9 × 10^7 | Cure              |             |
| Diseases | Patient ID | Age | Gender | Diagnosis basis | Region | Symptoms | Anti-rk39 | Etiology | Laboratory test | Imaging | Combined infections | Parasite load | Treatment outcome | Combined HPS |
|----------|------------|-----|--------|----------------|--------|----------|----------|----------|----------------|---------|---------------------|--------------|-------------------|-------------|
|          |            |     |        |                |        |          |          |          |                |         |                     |              |                   |             |
| 32       | 40         | M   | Iraq   | Multiple skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |
| 33       | 31         | M   | Iraq   | Multiple skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |
| 34       | 34         | M   | Iraq   | Multiple skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |
| 35       | 42         | M   | Iraq   | Multiple skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |
| 36       | 40         | M   | Iraq   | Multiple skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |
| 37       | 33         | M   | Iraq   | Multiple skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |
| 38       | 26         | M   | Nigeria| Multiple skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |
| 39       | 51         | M   | Iraq   | Multiple skin ulcers | +      | +        | +        | +        | +              | +       | +                   | +            | +                 | +            |
| 40       | 34         | M   | Iraq   | Multiple skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |
| 41       | 36         | M   | Uzbekistan | Single skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |
| 42       | 32         | M   | Iraq   | Multiple skin ulcers | −      | −        | −        | −        | −              | −       | −                   | −            | −                 | −            |

WBC: white blood cell; RBC: red blood cell; PLT: platelet; ALB: albumin; GLB: globulin; Cure means PCR negative in bone marrow for leishmania detection at the end of treatment; HPS: hemophagocytic syndrome; M: male; F: female; +: positive; −: negative; HBV: hepatitis B virus; un: undetected
L. tropica); third, gene fragments can obtain from NCBI database among different species and strains (Additional file 1: Table S1).

Each gene sequence among different species of Leishmania parasites were analyzed using MLSTest software (v1.0.1.23, institute de Patologia experimental Universidad Nacional de Salta Argentina, Boston, MA, USA), individually, and genes with sequence polymorphisms and species-specific SNPs were screened out (Additional file 2: Table S2). Furthermore, these sites with species-specific SNPs that can be completely distinguished Leishmania species which were selected, specifically, the optimal site that can identify species between L. donovani and L. infantum for VL and distinguishing species among L. major, L. tropica and L. donovani/L. infantum for CL were selected as targets (Figs. 1 and 2).

**Primers and probes design and plasmids construction**

Twenty-nine HGPRT sequences from nine different species of Leishmania parasites and 43 SPDSYN sequences from 18 different Leishmania parasites were collected from NCBI database and aligned using BIOEDIT software (v7.0.1, Ibis Biosciences, Carlsbad, CA, USA). Primers were designed based on the conserved region of sequence and probes were designed based on regions with species-specific SNPs of HGPRT genes between L. donovani and L. infantum and species-specific SNPs of SPDSYN genes among L.

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**Fig. 1** Alignment of HGPRT gene fragment sequences from nine Leishmania species with illustrations of primers and probes using for VL species identification between L. donovani and L. infantum. HGPRT Hypoxanthine-guanine phosphoribosyl transferase, VL Visceral leishmaniasis
major, L. tropica and L. donovani/infantum using PRIMER EXPRESS 3.0 (Applied Biosystems-Roche, Branchburg, America) (Table 2). The sequences of the designed primers and probes were tested against the NCBI nucleotide database using the BLASTn (Basic Local Alignment Search Tool) to confirm the species specificity.

DNA extraction
DNA was extracted from 200 µl bone marrow or 20 mg skin lesion tissue using a DNeasy Blood & Tissue Kit (Qiagen, 69506, Hilden, Germany) according to manufacturer’s instructions and DNA was stored at –20 °C.
Table 2  Sequence of primers and probes for the real-time PCR for CL and VL identification

| Diseases       | Target gene | Species                        | Primer and probe       | Sequences                                      | Amplicon size (bp) | GeneBank accession no. |
|----------------|-------------|--------------------------------|------------------------|------------------------------------------------|--------------------|------------------------|
| CL             | SPDSYN      |                                | CL-SPD-F               | 5’-AGATCATTGGCTACTTGAC-3’                      | 202                |                        |
| L. major       |             |                                | CL-SPD-R               | 5’-TCATCGACACACGACGAC-3’                      |                    |                        |
| L. tropica     |             |                                | P-SPD-LM               | 5’-VIC-TCAAGAGCCCCATCATCT-MGB-NFQ-3’           |                    |                        |
| L. infantum/infantum |             |                                | P-SPD-LDI              | 5’-Texas Red-TCAAGAGCCCCATCATCA-MGB-NFQ-3’     |                    |                        |
| VL             | HGPRT       |                                | VL-HGPRT-F2            | 5’-AGAAGATTGACAGAAGCT-3’                      | 145                |                        |
| L. donovani    |             |                                | VL-HGPRT-R1            | 5’-AACCTCCACCTCCGACAGACA-3’                    |                    |                        |
| L. infantum    |             |                                | P-HGPRT-LD             | 5’-FAM-ACAGAGTTTTAGGGTGACGACC-MGB-NFQ-3’      |                    |                        |
|                |             |                                | P-HGPRT-LI             | 5’-VIC-ACAGAGTTTTAGGGTGACGACC-MGB-NFQ-3’      |                    |                        |

Positive control plasmid construction
The HGPRT fragment of L. donovani and L. infantum, and SPDSYN fragment of L. major and L. donovani/infantum were amplified from identified clinical specimens and the fragment purified with DNA purification kit (TIANGEN, DP214, Beijing, China). The amplified HGPRT and SPDSYN fragments were ligated into plasmid pUC19 (TAKARA, 3219, Tokyo, Japan) using EcoRI and Hind III sites, individually. The correct cloning of the desired target DNA in the recombinant plasmid was confirmed by PCR amplification and DNA sequencing. Due to lack of L. tropica parasite and clinical samples from patients with L. tropica infection, SPDSYN fragment of L. tropica was synthesized based on sequence (Accession no. KM086079) and ligated in plasmid pUC19 by Sangon Biotech Co., Ltd, and then confirmed by PCR amplification and DNA sequencing.

An allele-specific real-time PCR assay for identification of Leishmania parasites
The allele-specific real-time PCR was conducted in a 20 μl reaction volume. For VL species identification, a reaction containing 10 μl of Promega GoTaq Probe qPCR Master Mix (Promega, A6101, Madison, WI, USA), 800 nmol/l forward primer VL-HGP-F2, 800 nmol/L reverse primer VL-HGP-R1, 450 nmol/L hydrolysis Probe P-HGP-LD (5’FAM/3’MGB-NFQ) and P-HGP-LI (5’VIC/3’MGB-NFQ), individually, plus 1 μl template DNA (5–50 ng). While for CL species identification, a reaction containing 10 μl of Promega GoTaq Probe qPCR Master Mix, 300 nmol/l forward primer CL-SPD-F, 300 nmol/L reverse primer CL-SPD-R, 450 nmol/L hydrolysis probes P-SPD-LM (5’VIC/3’MGB-NFQ), P-SPD-LT (5’FAM/3’MGB-NFQ) and P-SPD-LDI (5’Texas Red/3’MGB-NFQ), respectively, plus 1 μl template DNA (5–50 ng). The reaction was performed in the Applied Biosystems 7500 Fast real-time PCR System (ABI) with 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s, 62 °C (VL) and 58 °C (CL) for 50 s. Each sample was tested with replicates, the plasmid constructed above were used as positive control and reaction without template DNA (distilled water) was used as negative control in all experiments.

Analytical sensitivity and specificity of the allele specific real-time PCR for identification of Leishmania species
The limit of detection (LOD) of the allele-specific real-time PCR assay was defined as the minimum number of parasites that could be detected based on 8 repeated tests. We used cultured L. infantum promastigotes enumerated under a microscope and diluted with blood obtained from healthy volunteer as 1,000, 100, 50, 25, 12, 6, 3 or 1 parasites/μl. Total DNA was extracted from each dilution. The LOD was defined based on the experimentally derived assay precision (intra-assay SD < 0.5 and inter-assay CV <5%). The specificity of the allele-specific real-time PCR assay was tested with other DNA samples obtained from P. falciparum, T. gondii, B. melitensis and O. tsutsugamushi.

Two plasmids HGPRT/pUC19 of L. donovani and L. infantum and three plasmids SPDSYN/pUC19 of L. major, L. tropica and L. donovani/infantum were serial dilution as 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 copies/μl, individually. For testing the ability of identification among different species, and the PCR reaction efficiency
Development of allele specific real-time PCR assay for *Leishmania* species identification

To verify the potential application of HGPRT and SPDSYN in *Leishmania* species identification, the primers and probes were designed according to the conserved sequence of HGPRT and SPDSYN and the SNPs screened out above (Table 2).

Firstly, PCRs were performed with template from clinical samples or constructed plasmids. As expected, the primers, VL-HGPRT-F2 and VL-HGPRT-R1 for HGPRT and CL-SPD-F and CL-SPD-R for SPDSYN, can amplify a 145 bp fragment from *L. donovani*, *L. infantum* and *L. major* samples, and 202 bp fragment from *L. major*, *L. tropica*, *L. donovani* and *L. infantum* samples respectively. In addition, these two pair of primers didn't recognize any DNA from samples of *P. falciparum*, *T. gondii*, *B. melitensis* and *O. tsutsugamushi* (Fig. 3). These results indicated that the targets we selected here were specific for *Leishmania* species detection, which were potentially appropriate for further allele-specific real-time PCR assay construction.

Then an allele specific real-time PCR assay for *Leishmania* species identification were established using the primers and probes described above. Our results showed that this assay can detect 3 parasites/reaction for VL by targeting at HGPRT and 12 parasites/reaction for CL with SPDSYN (Additional file 3: Table S3).

The standard curves of this assay were also obtained using serially diluted plasmid DNA. It showed the PCR efficiency with both single-species and multi-species samples reactions were similar and the amplification curve were coincident as well (Fig. 4). The linear were over a 7-log range with a correlation coefficient (R²) of 0.995–0.999 for VL (Fig. 4A and B) and 6/7-log range with a R² of 0.994–0.999 for CL (Fig. 4C, D and G).

Moreover, both intra-CV% and inter-CV% of Ct values for 20 replicates were < 2% (Additional file 4: Table S4). All these results implied that this allele-specific real-time PCR assay exhibited high precision for VL and CL species identification.

Validation the established *Leishmania* species identification assay

As the allele-specific real-time PCR assay we developed above exhibited high PCR efficiency and precision, total 42 clinical samples were used to validate the performance of this assay (Table 3). For 22 clinical VL samples, the new method detected 3 as *L. donovani* infections and 19 as *L. infantum*, which was consistence with the sequencing results. Similarly, 20 skin lesion CL samples were all identified as *L. major* using by this new method and confirmed by sequencing as well.

Results

Targets selection for identification of *Leishmania* species

According to the inclusion criteria described in “Material and methods”, 21 genes were screened out from 34 genes, which were previously reported to exhibit sequence polymorphism among *Leishmania* species (Additional file 1: Table S1). Further analysis indicated that the identity of these 21 genes were 88.3–99.8% among different species and total 1,970 polymorphism sites were observed within them (Additional file 2: Table S2). Our further bioinformatics analysis were performed to select appropriate SNPs from these 1,970 polymorphism sites for *Leishmania* species identification. The alignment of 29 sequences of HGPRT from nine *Leishmania* species indicated that two SNPs can distinguish between *L. donovani* and *L. infantum* (Fig. 1). Moreover, 1–2 SNPs were found by comparison of 43 sequences of SPDSYN from 18 *Leishmania* species, which can distinguish *Leishmania* species among *L. major*, *L. tropica* and *L. donovani/L. infantum* well (Fig. 2). Thus, two potential targets for *Leishmania* species identification, HGPRT and SPDSYN, were screened out for further investigations.
A phylogenetic tree was constructed using 29 *Leishmania* HGPRT sequences (145 bp) from nine *Leishmania* species and 22 VL clinical samples. The clustering results show that 3/22 clinical samples (patient ID 10, 11, 19) were clustered with *L. donovani* and 19/22 clinical samples were clustered with *L. infantum* (Fig. 5 and Table 3). Also, phylogenetic analysis with 43 SPDSYN gene sequences (202 bp) from 18 *Leishmania* species and 20 CL clinical samples indicated that 20 clinical samples were all clustered with *L. major* (Fig. 6 and Table 3). Both of these two clustering outcomes were consistent with the new methods we developed here, which further confirmed the reliability of this new assay for *Leishmania* species identification.

**Discussion**

In this study, HGPRT and SPDSYN genes, which exhibit species-specific SNPs, were selected based on the screening of 21 housekeeping gene sequences from 9 species of VL and 18 species of CL. According to the conserved regions and species-specific SNPs, primers and probes were designed to perform two allele specific real-time PCR assays respectively. Our results showed that this new developed assay could identify the *Leishmania* species for VL between *L. donovani* and *L. infantum* with HGPRT and for CL among *L. major*, *L. tropica* and *L. donovani/L. infantum* with SPDSYN.

Previous studies identified *Leishmania* species using a SYBR-green based qPCR followed by melting analysis. Several different target were in these assays, including ITS1 for *Leishmania* (*Viannia*) spp., *L. donovani* complex, *L. tropica*, *L. mexicana*, *L. amazonensis*, *L. major*, and *L. aethiopica* [33]; canine beta-2-microglobulin and human glyceraldehyde-3 phosphate dehydrogenase for *Leishmania* (*Viannia*) spp., *L. infantum* and *L. amazonensis* [34]; amino acid permease 3 and cytochrome oxidase II (COII) genes for *L. major*, *L. tropica* and mix infections [35]; minicircle kDNA for the subgenera *Leishmania* and *Viannia* [36]; Cyt b gene for *L. braziliensis*, *L. guyanensis*, *L. infantum*, *L. tropica* and *L. panamensis* [27], and glucose-6-phosphate dehydrogenase for *L. braziliensis* or *L. peruviana* from the other *Leishmania* (*Viannia*) spp [32]. Although this type of assay was simple and cost-consuming, it is less specific and the results analysis was more complicated compared to the probe-based real-time PCR [25, 37].

There are also some real-time PCR identification methods were developed with different detecting targets, such as cathepsin L-like cysteine protease B gene for *L. major*, *L. tropica* and *L. aethiopica* [38]; amino acid permease 3 (AAP3) and COII for *L. major* and *L. tropica* [39], and glucose phosphate isomerase (GPI) for *Leishmania* (*Viannia*) spp., *L. mexicana* complex, *L. infantum/donovani* complex and *L. major* complex [31]. The two allele-specific qPCR assays we developed here were focused on the *Leishmania* species that are common in clinical practice, such as *L. donovani*, *L. infantum*, *L. major* and *L. tropica*. Using two firstly reported targets, HGPRT and SPDSYN genes with species-specific SNPs, the LOD of these assays was 3.
and 12 parasites/reaction for VL and CL, individually and no cross-reaction with *P. falciparum*, *T. gondii*, *B. melitensis*, *O. tsutsugamushi* and non-target species of *Leishmania* was detected (Additional file 3: Table S3; Figs. 4 and 5). Considering it takes only 2.5 h to identify *Leishmania* species directly from clinical samples without parasites isolation or culture, these assays are suitable in clinical practice.

A total of 42 clinical samples (22 VL and 20 CL) were used to evaluate the performance of the allele-specific real-time PCR assay, which identified 22 VL clinical samples as *L. donovani* (*n* = 3) and *L. infantum* (*n* = 19), 20 CL clinical samples as *L. major* (*n* = 20). These results were consistent with the following sequencing analysis, which indicated that these new tools can distinguish SNPs among different *Leishmania* species well (Table 3). Further phylogenetic analysis was performed to validate the results of these allele-specific qPCR assays, which confirmed their reliability for potential clinical applications (Figs. 5 and 6).

HGPRT gene encoded hypoxanthine phosphoribosyl transferase, which is a central enzyme in the purine recycling pathway of all protozoan parasites [40]. Spermidine synthase encoded by SPDSYN gene is a key enzyme in the polyamine biosynthetic pathway of protozoan parasites [41]. These two housekeeping gene sequences exhibit observed interspecies polymorphism, which imply that our assays in this study could be applied to distinguish not only *Leishmania* species we described here, but also other species not included in this study. Indeed, our phylogenetic analysis implied that the sequence of HGPRT gene could differentiate more *Leishmania* species than we tested here, including *L. major*, *L. mexicana* complex and *Leishmania* (*Viannia*) subgenus (Fig. 5). Meanwhile, SPDSYN gene fragment appears to be able to distinguish *Leishmania* (*Viannia*) braziliensis, *L. mexicana* complex and *Leishmania* (*Viannia*) subgenus as well (Fig. 6). Further investigations are worthwhile to be performed to extend the potential scope of these identification assays.

Broad variations are noted in efficacies of leishmaniasis treatment depending on the *Leishmania* species, which identification would be helpful in clinical practice. For example, antimonial and miltefosine are more effective to *L. major* and *L. donovani* than *L. infantum* [16, 42, 43]. Unlike *L. major*, *L. tropica* appears unresponsive to PM-based ointments [44, 45]. Amphotericin B is used to treat *L. tropica* or *L. major* related CL, but not *L. infantum* [46–48]. The efficacy rates of azoles for *L. infantum*, *L. donovani*, *L. major* and *L. tropica* were 88%, 80%, 53% and 15%, respectively [49]. Further, *Leishmania* species-specific administrations were applied for better clinical efficiency. For *L. tropica* infection, intralesional treatment was more efficient than intramuscular administration with sodium stibogluconate [50]. Intravenous antimonial treatment could produce better cure rates against *L. panamensis* or *L. braziliensis* related CL compared with *L.
| Diseases            | Patients ID | Testing results Ct (SD) | Sequencing results | Diseases | Patients ID | Testing results Ct (SD) | Sequencing results |
|---------------------|-------------|-------------------------|--------------------|----------|-------------|-------------------------|--------------------|
| L. donovani         | 1           | un                      | 30.7 (0.3)         | L. infantum | 23          | 30.1 (0.3)              | un                 |
| L. infantum         | 2           | un                      | 34.4 (0.1)         | L. infantum | 24          | 300 (0.3)               | un                 |
| L. major            | 3           | un                      | 34.8 (0.3)         | L. infantum | 25          | 264 (0.1)               | un                 |
| L. major            | 4           | un                      | 33.7 (0.2)         | L. infantum | 26          | 282 (0.2)               | un                 |
| L. major            | 5           | un                      | 33.2 (0.2)         | L. infantum | 27          | 297 (0.2)               | un                 |
| L. major            | 6           | un                      | 36.8 (0.4)         | L. infantum | 28          | 323 (0.3)               | un                 |
| L. major            | 7           | un                      | 27.1 (0.1)         | L. infantum | 29          | 356 (0.4)               | un                 |
| L. major            | 8           | un                      | 31.0 (0.1)         | L. infantum | 30          | 262 (0.1)               | un                 |
| L. major            | 9           | un                      | 33.8 (0.2)         | L. infantum | 31          | 256 (0.1)               | un                 |
| L. major            | 10          | 29.43 (0.16)            | un                 | L. donovani | 32          | 272 (0.2)               | un                 |
| L. major            | 11          | 27.66 (0.15)            | un                 | L. donovani | 33          | 323 (0.4)               | un                 |
| L. major            | 12          | 27.0 (0.3)              | un                 | L. donovani | 34          | 294 (0.2)               | un                 |
| L. major            | 13          | 35.9 (0.4)              | un                 | L. donovani | 35          | 276 (0.2)               | un                 |
| L. major            | 14          | 31.7 (0.3)              | un                 | L. donovani | 36          | 283 (0.4)               | un                 |
| L. major            | 15          | 36.1 (0.5)              | un                 | L. donovani | 37          | 283 (0.6)               | un                 |
| L. major            | 16          | 38.5 (0.4)              | un                 | L. donovani | 38          | 298 (0.1)               | un                 |
| L. major            | 17          | 33.4 (0.7)              | un                 | L. donovani | 39          | 288 (0.2)               | un                 |
| L. major            | 18          | 26.4 (0.1)              | un                 | L. donovani | 40          | 264 (0.4)               | un                 |
| L. major            | 19          | 34.45 (0.39)            | un                 | L. donovani | 41          | 291 (0.4)               | un                 |
| L. major            | 20          | 31.2 (0.1)              | un                 | L. donovani | 42          | 35.0 (0.4)              | un                 |
| L. major            | 21          | 29.2 (0.3)              | un                 | L. infantum |             |                        |                    |
| L. major            | 22          | 25.3 (0.3)              | un                 | L. infantum |             |                        |                    |

Ct: cycle threshold, SD: standard deviation, VL: visceral leishmaniasis, CL: cutaneous leishmaniasis, un: undetected
Fig. 5 Neighbor-Joining was used to generate phylogenetic tree with 1000 replications for bootstrap based on HGPRT gene fragment, including sequences from patients' samples.
Fig. 6 Neighbor-Joining was used to generate phylogenetic tree with 1000 replications for bootstrap based on SPDSYN gene fragment, including sequences from patients' samples.

- **L. major complex**
- **L. tropica complex**
- **L. donovani complex**
  - **L. viannia braziliensis**
- **L. mexicana complex**
- **L. viannia subgenus**

**Legend:**
- **L. major**
- **L. tropica**
- **L. donovani**
- **L. viannia**
- **L. mexicana**
major [51–53]. Thus, as a rapid and accurate tool for Leishmania species identification, it would be helpful for species-adapted therapeutic schedule and patient management.

Unfortunately, MLEE, the “gold standard” method for Leishmania species identification, could not be performed in this study, due to only a few of parasites can be cultured in vitro from our stored clinical samples. Instead of MLEE, phylogenetic analysis of HGPRT and SPDSYN was applied to further confirm our species distinguish results. Although our new methods with HGPRT and SPDSYN can distinguish between L. donovani and L. infantum for VL and among L. major, L. tropica and L. donovani/infantum of CL accurately, a larger sample size should be investigated in future for further confidence, especially with clinical samples of L. tropica infection and different species co-infection which were not applied in this study.

Conclusions
A novel probe-based allele-specific real-time PCR assay was established with newly reported targets, HGPRT and SPDSYN, which could identify Leishmania species between L. donovani and L. infantum for VL, and among L. major, L. tropica and L. donovani/infantum for CL. This method could be applied for not only Leishmania species-adapted therapeutic management but also ecological and epidemiological studies.

Abbreviations
VL: Visceral leishmaniasis; CL: Cutaneous leishmaniasis; ML: Mucosal leishmaniasis; PKDL: Post kala-azar dermal leishmaniasis; RFLP: Restriction fragment length polymorphism; MLEE: Multi-site enzyme electrophoresis; HGPRT: Hypoxanthine-guanine phosphoribosyl transferase; SPDSYN: Spermidine synthase; SNPs: Single nucleotide polymorphisms; LOD: Limit of detection; AAP3: Amino acid permease 3, COII: Cytochrome oxidase II; GPI: Glucosephosphate isomerase; PM: Paromomycin.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s40249-022-00992-y.

Additional file 1: Table S1. 34 housekeeping genes of Leishmania with sequence polymorphism.

Additional file 2: Table S2. Leishmania interspecies polymorphism in 21 genes.

Additional file 3: Table S3. The sensitivity of allele-specific real-time PCR assay.

Additional file 4: Table S4. Precision of intra and inter-assay of allele-specific real-time PCR assay.

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Author contributions
YW, GY designed the study and interpreted the findings. YW, MJ and SL contributed to data collection and validation. YW conducted data analysis and writing of this original draft. GY and NRW review and editing. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
This project has been approved by the Ethics Committee of Beijing Friendship Hospital (Beijing, China) with approval number of 2021-P2-356-01. All clinical samples investigated in this study were obtained from an existing sample collection. All samples were anonymized.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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