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Original Article

Assessment of pan-*Leishmania* detection by recombinase polymerase amplification assay

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ABSTRACT

The spread of vector habitats along with increasing human mobility can introduce atypical *Leishmania* species and hence can challenge existing diagnostic practices for rapid detection of active infection with species outside the narrow target range. Here we assessed the pan-*Leishmania* detection ability of isothermal recombinase polymerase amplification (RPA) assays targeting *18S rRNA* gene, cathepsin L-like cysteine proteinase B (*Cpb)* gene, and kinetoplast minicircle DNA (kDNA) regions. While the lowest limit of detection of the 18S rRNA-RPA and Cpb-RPA assays were estimated as 12 and 17 standard DNA molecules, respectively, both assays could amplify genomic DNA of 7 pathogenic *Leishmania* species. Evaluation of 18S rRNA-RPA and our previously developed kDNA-RPA assays on 70 real-time PCR-positive leishmaniasis samples of varying pathologies resulted in sensitivity rates of 35.71% and 88.57%, respectively, while the combined sensitivity was 98.57%. Combinatorial application of 18S rRNA-RPA and kDNA-RPA assays can be recommended for further diagnostic assessments.

1. Introduction

Leishmaniasis is a disease caused by obligate intracellular protozoan and flagellated parasites of the genus *Leishmania*, belonging to the family *Trypanosomatidae*, order *Kinetoplastidia*. The parasite is transmitted to humans *via* the bite of infected female sandflies of the genus *Phlebotomus* and *Lutzomyia* (E. K. Elmahallawy et al., 2014). Leishmaniasis is endemic in nearly 100 countries (Murray HW et al., 2005). They are geographically divided into 2 groups: "New World" leishmaniasis - typically found in America - and the "Old World" leishmaniasis, which is endemic in several tropical countries of Asia and Africa, and also in countries of the Mediterranean region Herwaldt, 1999; Piscopo and Mallia Azzopardi, 2007. However, the spread of endemic areas owing mostly to the change in sandfly habitats and human mobility are of recent concern (Ferreira et al., 2001).

The main clinical forms of leishmaniasis are cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), visceral leishmaniasis (VL), and its dermal complication called post-Kala-azar dermal leishmaniasis (PKDL) (E. K. Elmahallawy et al., 2014). CL is most commonly caused by *L. major, L. tropica, L. aethiopica, L. amazonensis, L. mexicana, L. braziliensis, L. panamensis, and L. guyanensis,* depending on the geographical spread of the species in endemic regions Desjeux, 2004; Ready, 2010. Common pathological consequences include various skin manifestations such as ulcerations covered by crusts, raised margins, and erythematous papules, plaques, and nodules. Mucocutaneous leishmaniasis (MCL) can be caused by *L. braziliensis, L. panamensis,* and *L. guyanensis* and is developed often after CL (Grevelink and Lerner, 1996). Visceral leishmaniasis (VL) or "Kala-azar" caused by the *L. donovani* complex is the most fatal form of leishmaniasis if left untreated and is highly endemic in the Indian subcontinent and in East Africa (Herwaldt, 1999). Parasite infection in

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viscerotropic organs can lead to splenomegaly, pancytopenia, hepatomegaly, fever, weight loss, and mucosal bleeding. PKDL is an atypical and chronic dermatosis that often develops as sequelae of VL - mostly after treatment. PKDL manifestation is in the form of painless macular or papulo-nodular lesions or a mix of both that harbor parasites. Another less common type is diffuse cutaneous leishmaniasis (DCL) caused by *L. amazonensis* and *L. aethiopica* (E. K. Elmahallawy et al., 2014), which features typical nodular or plaque-shaped solitary lesions.

The diagnostic protocol for leishmaniasis involves a provisional diagnosis, which is based on clinical presentation and symptoms, usually followed by a confirmatory test, which is usually either culture, microscopy, or polymerase chain reaction (PCR). Cases of leishmaniasis may pose a diagnostic dilemma as the signs and symptoms are often similar to those of other infectious and/or genetic diseases, particularly in imported cases and for species of *Leishmania*, which are nonendemic at a particular region. For instance, pathological manifestations of VL often mimic symptoms commonly resulting from bacterial and other protozoan parasites (e.g., tuberculosis, toxoplasmosis), viral infections, malignancies (e.g., leukemia, lymphoma), liver cirrhosis and autoimmune disorders such as Systemic Lupus Erythematosus and autoimmune hepatitis (Bogdan, 2012; Jones et al., 2003; Santana et al., 2015; Tunccan et al., 2012). Differential diagnosis of PKDL usually includes leprosy, vitiligo, and miliaria rubra (n. d.) while the clinical appearance of CL and MCL often resembles bacterial (lupus vulgaris) and fungal (sporotrichosis) infections, autoimmune disorders (Localized Lupus Erythematosus), various cancers (oral carcinoma, T-cell lymphoma, squamous cell carcinoma) and tumors, as discussed elsewhere (Schwing et al., 2019).

In such cases, parasite detection using culture and/or microscopy could be limiting for sensitivity (Garcia et al., 2005), whereas PCR methods are mostly targeted for only one species or a particular form of leishmaniasis. Moreover, antibody detection tests such as the rk39 rapid diagnostic test largely cannot differentiate between active infection and previous episodes Srividya et al., 2012; E. Elmahallawy et al., 2014, while antileishmanial IgE, IgM, and IgG have shown promise for detecting recent infection (Fagundes-Silva et al., 2012; Kumar and Nylén, 2012; Ozbılge et al., 2006). On the other hand, intradermal test (also called leishmanin skin test), which is possible for CL or MCL is about 95% sensitive and 96% to 100% specific, however, it cannot diagnose VL. Of note, leishmanin antigen is no longer produced under good manufacturing practice conditions anywhere in the world. Consequently, the use of skin tests has declined in favor of serological and molecular tests (Carstens-Kass et al., 2021).

PCR is known to be more sensitive, and to be a specific and robust test for detection of active infection (Nicolas et al., 2002; Stevenson et al., 2010; Tupperwar et al., 2008; Wortmann et al., 2005), however, it is limited to laboratories with relatively complex technical equipment (A Abd El Wahed et al., 2015). A low-cost and easy to establish simple molecular diagnostic method such as recombinase polymerase amplification (RPA) would leverage the setup at the often resource-poor settings for the detection of *Leishmania spp* in endemic countries. RPA runs significantly faster (15−20 minutes) and at a constant temperature of about 37 to 42°C to produce results that demonstrated sensitivity and specificity comparable to PCR-based methods. The unique feature of the RPA amplification lies in the formation of recombinase enzyme-primer complexes that upon homology in a double-stranded DNA template, performs strand-invasion to insert the primer sequence. In this way, the strand-displacing polymerase can ultimately bind to the primer and synthesize the desired amplicon (Piepenburg et al., 2006). For real-time detection, an exo-probe is used and the emitted fluorescence signal can be measured. The lyophilized pellets of the enzyme mix are relatively stable in ambient temperature requiring no cold-chain maintenance, which facilitates its operation in mobile settings in a portable suitcase (A. Abd El Wahed et al., 2015).

Several RPA assays have been developed to date that are either specific for one or a few species of *Leishmania*. We have previously developed RPA assay targeting the kDNA, which was found to be very sensitive and specific for the detection of *L. donovani* that typically causes visceral- and post-Kala-azar dermal leishmaniasis, and cross-reactive to *L. major, L. aethiopica* and *L. infantum* but not the other infectious species (Mondal et al., 2016). Furthermore, this qualitative RPA assay was found to have absolute agreement with an established real-time qPCR assay in terms of sensitivity and specificity, and so had the quantitative RPA assay that used the same target (Kha et al., 2021). The aim of the present study was to develop a pan-*Leishmania* RPA assay for the detection of all *Leishmania* pathotypes. Two RPA assays targeting the *18S ribosomal RNA* gene- (18S rRNA-RPA assay) and the cathepsin L-like cysteine proteinase B (*Cpb*) gene (Cpb-RPA assay) were developed. Their ability to detect common pathogenic species was tested, and the better performer between the assays was selected for clinical validation.

2. Materials and methods

2.1. Sample source and ethical consideration

Archived DNA samples extracted from clinical specimens were retrieved upon approval from the Ethical Review Committee of the International Centre for Diarrheal Disease Research, Bangladesh (PR-18023), Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka (787/13 & 15/18) and the University Medical Center Goettingen, Germany (26/9/2018). A total of 100 archived DNA samples were included in the study, 70 of which originated from leishmaniasis patients (25 VL, 25 PKDL, and 20 CL) and the rest from apparently healthy subjects from endemic areas. All of the study participants had consented for use of archived samples in future research needs.

2.2. DNA molecular standards and genomic DNA

Molecular DNA standards representing 300 bases of gene segment of the *Cpb* gene (GenBank accession number: DQ286773.1, nt 439-739) and 597 bp of the *18S rRNA* gene, (GenBank accession number: XR_002966730.1, nt 404-1001) were synthesized by GeneArt (Life Technologies, Darmstadt, Germany). Dilution series ranging from 10^7 to 10^0 molecules/μL were prepared from each of the molecular standards to determine the analytical sensitivity of Cpb-RPA and 18S rRNA-RPA assays. Genomic DNA isolated from cell cultures of various *Leishmania* species (Supplementary Table S1) was used to determine the species coverages. DNA of other pathogens were screened to assess the cross-reactivity of the assay (Supplementary Table S2).

2.3. RPA oligonucleotides

Primers and probes were designed by using MEGA7 (Kumar et al., 2016) and GENEIOUS (Biomatter Ltd.; Auckland, New Zealand) programs. For this study, 6 primers and one exo-probe for the Cpb-RPA assay, and eight primers and 1 exo-probe for the 18S rRNA-RPA assay (Table 1) were tested to select combinations that produce the highest assay analytical sensitivity. In addition, primers and exo-probe sequences of our previously developed RPA assay that targets kinetoplast minicircle DNA (kDNA) Mondal et al., 2016; Kha et al., 2021 were tested to evaluate the diagnostic performance of the kDNA-RPA assay in archived DNA samples extracted from VL, PKDL and CL clinical specimens mentioned above. All oligonucleotides were synthesized by Tib MolBiol (Berlin, Germany).

2.4. RPA assay conditions

The RPA reaction was performed in a volume of 50 μL using a Twist-Amp exo kit (TwistDx Ltd; Cambridge, UK) as per manufacturer recommendation. The master mix preparation contained 2.1 μL of each primer (10 μM), 0.6 μL exo-probe (10 μM), 29.5 μL rehydration buffer and 8.2 μL nuclease-free water for each reaction. Five microliters of DNA template were added along with master mix into the reaction tube containing the freeze-dried pellet. Finally, 2.5 μL of magnesium acetate was pipetted and this was followed by immediate mixing, short-spinning and incubation in tube scanner ESEQuant (QIAGEN Lake Constance GmbH; Stockach, Germany) or Axxin T8-ISO (Fairfield, Australia) reaction chamber at 42 °C. After 230 seconds, the reaction was removed from the scanner, mixed and centrifuged briefly and then put back in order to complete the scanning for a total of 15 minutes. Threshold and first derivative analysis calculated from the fluorescence signal intensities were used for signal interpretation. All RPA assay procedures were conducted inside mobile suitcases that contained all the apparatus needed to perform the assay (Piepenburg et al., 2006).

2.5. Real-time PCR

Real-time PCR was used as the gold standard. For VL and PKDL samples, real-time PCR was performed according to the procedure described previously (Vallur et al., 2014). The Bio-Rad CFX96 iCycler system was used with the following cycling conditions: 10 minutes at 95 °C, followed by 45 cycles of 15 seconds at 95 °C and 1 minutes at 60 °C. For CL samples, a real-time PCR assay as described by El Tai et al. was applied (El Tai et al., 2000). The QuantiTect SYBR Green PCR Kit (Qiagen; Hilden, Germany) was used as described by the manufacturer. Bio-Rad CFX96 real-time PCR Machine was adjusted to the following thermal cycling profile: 95 °C for 15 minutes, then 40 cycles of 95 °C for 20 seconds, 53 °C for 30 seconds, and 72 for 1 minute as well as melting curve analysis at 50 °C to 95 °C, increment of 0.5 °C for 5 seconds and a final cooling step at 25 °C for 30 seconds.

2.6. Statistical methods

To determine the analytical sensitivity of Cpb-RPA and 18S rRNA-RPA assays by using the molecular DNA standard, semilog regression analysis and probit analysis were performed by plotting the RPA threshold time against the number of molecules detected using PRISM (Graphpad Software Inc.; San Diego, California) and STATISTICA (StatSoft; Hamburg, Germany), respectively. Based on the probit model, an expected value is estimated at which 95% of the RPA runs are positive. In the semilogarithmic regression model, the detection time is determined from the results of eight RPA runs of samples with different concentrations. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated using standard formulas as mentioned previously (Parikh et al., 2008). Cohen's kappa measure of agreement (κ) was performed to determine the concordance in detection outcome between RPA assays and real-time PCR tests for archived clinical DNA samples.

Table 1
Oligonucleotides sequences.

Name	Sequenz (5′ – 3′)
Cpb RPA FP1	CTTGCCGGCCACGGGCTGACGGCCCTGTCG
Cpb RPA FP2	TCGGCAGCATCGAGTCGCAGTGGGCCCTTGC
Cpb RPA FP3	GTGCTGGGCGTTCTCGGCGGTCGGCAGCAT
Cpb RPA RP1	GAACGCCTGCAGCATCAGCCCGCCGCCGC
Cpb RPA RP2	ATGTTTCGCAGCAGCCACTCGAACGCCTGCAGCA
Cpb RPA RP3	TGTCCTCCGTGAACATGGTCCCGTTCATG
Cpb RPA P	AGCATCAGCCCGCCGCCGCAACCATTGTC (BHQ1-dT) (Tetrahydrofuran)(FAM-dT) GTCATCGCAGCTCA-Phosphate
18S rRNA-RPA FP1	TCAAGAACCAAAGTGTGGAGATCGAAGATGATTAG
18S rRNA-RPA FP2	TACAGCGAAGGCATTCTTCAAGGATACCTTCCTCA
18S rRNA-RPA FP3	GTCAGAGGTGAAATTCTTAGACCGCACCAAGACG
18S rRNA-RPA FP4	TTCGTCCGGCCGTAACGCCTTTTCAACTCACGGC
18S rRNA-RPA RP1	GCCCATAAGATCCCCAATTCATGGGTGTCATCG
18S rRNA-RPA RP2	GTGCTGACACAGGGTAAACCCTGCCGCAGGCCG
18S rRNA-RPA RP3	TGGTAAAAGCGGGCGCGGTGCTGACACAGGGTA
18S rRNA-RPA RP4	TGACACAGGGTAAACCCTGCCGCAGGCCGGCCCAT
18S rRNA-RPA P	TGTGGAGATCGAAGATGATTAGAGACCAT(BHQ1-dT) (Tetrahydrofuran)(FAM-dT) AGTCCACACTGCAA-Phosphate

3. Results

Several primers targeting *Cpb* and *18S rRNA* genes (Table 1) were screened using molecular DNA standards (10^5 molecules/μL) to select the best oligonucleotide combinations. For the Cpb-RPA assay, the combination of FP2 and RP1 produced the best amplification curve (Supplementary Fig. S1). For the 18S rRNA-RPA assay, most of the oligonucleotides combinations amplified the target gene (Supplementary Fig. S2), but FP4 and RP4 was highly specific to *Leishmania* spp and did not amplify *T. brucei* (Supplementary Fig. S3). On the other hand, all other primers cross-reacted with *T. brucei* (not shown).

In order to determine the analytical sensitivities of the RPA assays, eight assay runs on dilution series of the molecular standard (10^3–10^1 molecules/μL) were performed. The collected data were subjected to probit regression analysis. The Cpb-RPA assay estimated limit of detection was 17 DNA molecules with 95% probability, while for the 18S rRNA-RPA assay it was 12 DNA molecules (Fig. 1).

Promastigote DNA from culture of several *Leishmania* species was tested with both assays. The Cpb-RPA assay detected *L. major, L. donovani, L. infantum, L. aethiopica, L. tropica and L. amazonensis* but was less reactive to *L. braziliensis* (Table 2 and Supplementary Fig. S4). In contrast, all the test species were found positive with the 18S rRNA-RPA assay (Table 2 and Supplementary Fig. S5). To determine the cross-reactivity of the RPA assays to other pathogens, 14 non-*Leishmania* standard DNA samples were tested. None of the other bacterial and non-*Leishmania* parasites were detected by either of the RPA assays.

For the validation of the RPA test with clinical patient samples, the 18S rRNA-RPA was chosen because it showed the best preclinical performance among the 2 RPA assays. The performance of the 18S rRNA-RPA test was further evaluated using 100 archived DNA samples (70 *Leishmania*-positive and 30 negatives in real-time PCR). The kDNA-RPA assay Mondal et al., 2016; Kha et al., 2021 was also included for the clinical assessment of its pan-*Leishmania* performance. All 30 samples from uninfected and apparently healthy subjects were negative using the 18S rRNA-RPA and kDNA-RPA assays. However, only 1 of 25 samples from VL patients, 5 of 25 samples from PKDL patients, and 19 of 20 samples from patients with CL were positive with 18S rRNA-RPA assay. Poor performance against the VL and PKDL groups could be associated with low analytical sensitivity of the 18S rRNA-RPA assay in detecting genomic DNA of *L. donovani* (lower limit of detection: 100 parasite genome/reaction, Supplementary Fig. S6), which is the only endemic species known-to-date that cause VL and PKDL in Bangladesh. On the other hand, the kDNA-RPA assay detected the VL and PKDL samples with high sensitivity, but underperformed in case of CL samples. Nevertheless, 18S rRNA-RPA and kDNA-RPA assays could achieve cumulatively over 98% sensitivity for any form of leishmaniasis in the given pool of test samples (Table 3).

4. Discussion

Leishmaniasis is still defined as an endemic infection confined to certain areas across the world. However, climate changes accompanied by spreading of vector habitats and increased human mobility can contribute to worldwide transmission of pathogens (Rocklöv and Dubrow, 2020). In fact, a growing number of reports have been unveiling the existence of cases and/or species with no previous history of their occurrence in nonendemic locales [(Antinori et al., 2005;

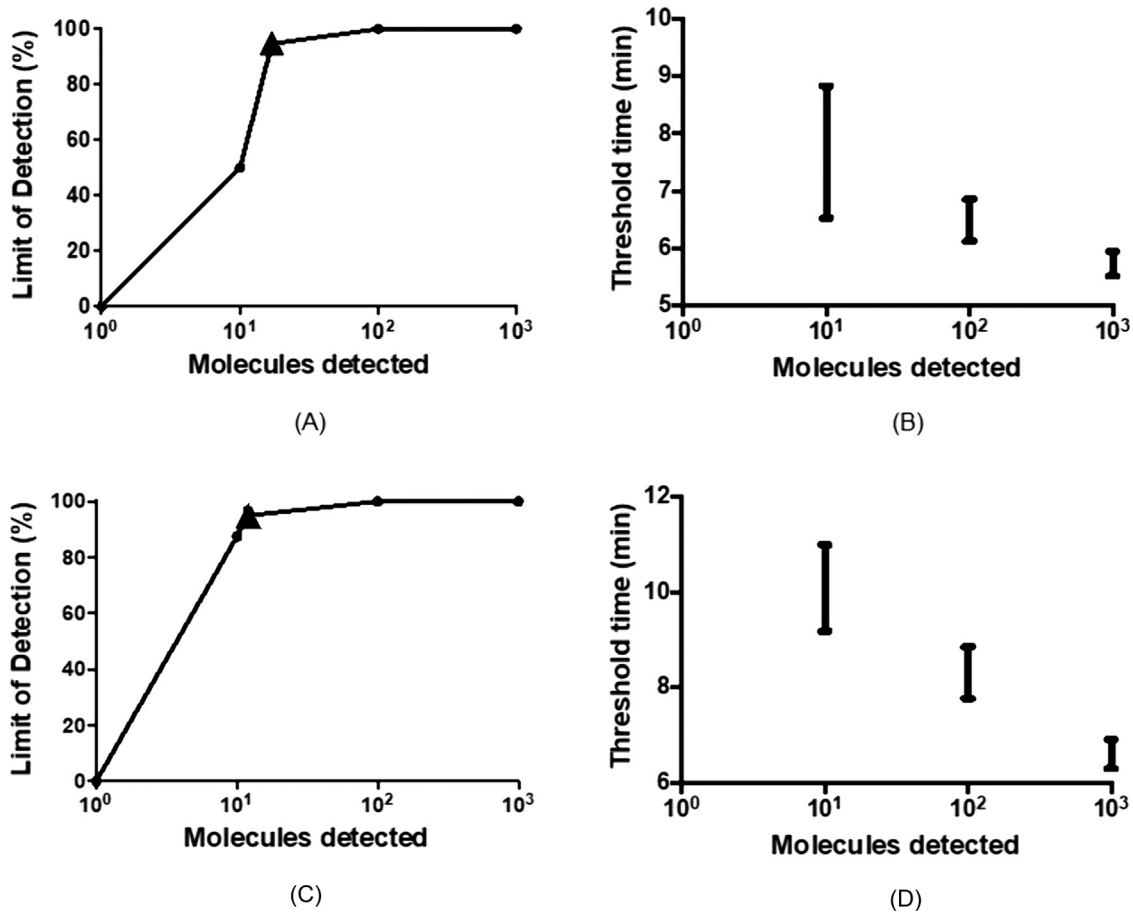

Fig. 1. Probit regression analysis and reproducibility for eight RPA assay runs using the DNA molecular standard. Cpb-RPA assay: (A) The limit of detection at 95% probability (17 DNA molecules) is depicted by a triangle. (B) Results was produced between 5 and 9 minutes and 10^3 to10^2 DNA molecules were detected in 8 of 8 runs, while 10^1 DNA molecules were identified in 4 of 8 runs. 18S rRNA-RPA assay: (C) The limit of detection at 95% probability (12 DNA molecules) is depicted by a triangle. (D) Results was produced between 2 and 12 minutes. 10^3 to10^2 DNA molecules were detected in 8 of 8 runs, while 10^1 DNA molecules were identified in 7 of 8 runs.

Pavli and Maltezou, 2010; Stark et al., 2008)]. In Bangladesh as well, where *L. donovani* which typically causes VL and PKDL is the only known causative and transmissible species to date, a number of reports demonstrated imported infections with CL-causing parasites [(Basher et al., 2017; Khan et al., 2019; Rahman et al., 2014)]. Nonspecificity of visceral and cutaneous forms of leishmaniasis regarding clinical symptoms and manifestations similar to other infectious/non-infectious diseases can lead to a diagnostic and therapeutic dilemma. From an epidemiological perspective, the introduction of and failure to identify a new species in an endemic area can complicate disease control maneuvers as well. While a definitive diagnosis of leishmaniasis still relies to some extent on parasitological confirmation by an expert, deployment of more reliable PCR-based assays is limited by laboratory capacity, cost, the feasibility of operation, availability, and species coverage. Also, in terms of diagnostic performance, considerable differences in accuracy exist among diagnostic modules, thus limiting their case-specific diagnostic appropriateness. Culture and microscopy are known to be less sensitive than molecular assays in detecting parasites in clinical/biological specimens (skin biopsy, peripheral blood, sand fly midgut etc.) Bahrami et al., 2018; Khan et al., 2021, while antigen and antibody-based assays can be inferior in sensitivity and specificity and/or incapable to differentiate active infection from past exposures [(Abeijon et al., 2019; Galluzzi et al., 2018; Mondal et al., 2019)]. This scenario dictates that a rapid, accurate and widely applicable molecular tool capable to detect any *Leishmania* infection of clinical importance is highly desirable. Here we took an approach to develop an isothermal RPA-based rapid pan-*Leishmania* assays targeting conserved regions of *18S rRNA* and *Cpb* genes. Evaluation of the assays by using molecular standards, culture-derived genomic DNA, and clinical samples corroborates their potential in rapid screening and sensitive and specific diagnosis of suspected cases.

Table 2
Leishmania species coverage in the *Leishmania* Cpb-RPA and 18S rRNA-RPA assays.

Pathogen	Number of isolates	RPA target 18S rRNA	Cpb
L. tropica	5	+	+
L. donovani	5	+	+
L. major	4	+	+
L. aethiopica	5	+	+
L. amazonensis	1	+	+
L. infantum	1	+	+
L. braziliensis	5	+	+/-

The symbol + denotes positive detection of all the isolates of a species, while +/- indicates poor resolution.

In *Leishmania* species, the *18S rRNA* gene is highly conserved and exists in between 50 and 200 copies per genome Srivastava et al., 2011; Tuon et al., 2008. Similarly, the polymorphic and multicopy *Cpb* gene that encodes for a major antigen (cathepsin L-like cysteine proteinase B) of *Leishmania* parasites is also conserved among the *Leishmania* species Kuru et al., 2011; Sakanari et al., 1997. Both of these genomic targets thus make ideal candidates for pan-*Leishmania* molecular assay and were assessed in the previous developments of several PCR-based assays for genus and species-specific detection

Table 3
Sensitivity, specificity, positive predictive value, negative predictive value and agreement (Kappa) of 18S rRNA-RPA and kDNA-RPA assays with real-time PCR method assessed by using archived clinical samples.

		18S rRNA-RPA	kDNA-RPA	Cumulative
Specificity [95% CI]	Healthy subjects (n = 30)	100% [88.43%–100.00%]	100% [88.43%–100.00%]	100% [88.43%–100.00%]
Sensitivity [95% CI]	VL (n = 25)	4% [0.10%–20.35%]	100% [86.28%–100.00%]	100% [86.28%–100.00%]
	PKDL (n = 25)	20% [6.83%–40.70%]	100% [86.28%–100.00%]	100% [86.28%–100.00%]
	CL (n = 20)	95% [75.13%–99.87%]	60% [36.05%–80.88%]	95% [75.13%–99.87%]
	Total disease case (n = 70)	35.71% [24.61%–48.07%]	88.57% [78.72%–94.93%]	98.57% [92.30%–99.96%]
Positive predictive value [95% CI]	All samples (n = 100)	100% [83.42%–100.00%]	100% [92.73%–100.00%]	100% [93.42%–100.00%]
Negative predictive value [95% CI]	All samples (n = 100)	40% [29.06%–51.97%]	78.95% [62.21%–89.86%]	96.77% [81.48%–99.83%]
Method agreement (κ-statistic with 95% CI)	vs Real-time PCR	0.25 [0.143–0.357]	0.823 [0.707–0.939]	0.976 [0.930–1]

Srivastava et al., 2011; Gow et al., 2019; Nath-Chowdhury et al., 2016. Furthermore, the high copy-number kinetoplast minicircle DNA (kDNA) is conserved among the different *Leishmania* species (Cantacessi et al., 2015), which corroborates the cross-detection of CL-causing species such as *L. major*, as observed in our previously developed RPA assay intended for the diagnosis of VL and PKDL in Bangladesh (Mondal et al., 2016). In this study, we designed new RPA assays based on the *18S rRNA* and *Cpb* gene regions conserved in the pathogenic *Leishmania* species. Respectively, three and 4 pairs of primer sets were tested around exo-probe binding sites of the target segments of *Cpb* and *18S rRNA* genes. Only the FP2-RP1 combination produced a high fluorescence signal in the Cpb-RPA assay, while several primer combinations targeting the *18S rRNA* gene region were equally effective in producing signals. However, unlike other primer combinations that were tested, it was only the FP4-RP4 combination that did not amplify the *T. brucei* genomic DNA and thus was selected for further evaluation. Both the 18S rRNA-RPA and Cpb-RPA assays detected all the old and new world pathogenic *Leishmania* species included in the study (i.e., *L. major, L. donovani, L. infantum, L. aethiopica, L. tropica, L. amazonensis,* and *L. braziliensis*), with the latter assay providing poor resolution in case of *L. braziliensis*. None of the 14 non-*Leishmania* parasites (including *Trypnosoma, Toxoplasma,* and *Plasmodium* species) and bacteria species were detected by either of the assays. Respective assays were estimated to detect 12 and 17 copies of the molecular DNA standards of *18S rRNA* and *Cpb* target regions with 95% sensitivity. Furthermore, repeated assays 8 times with as low as 10 copies of *18S rRNA* and *Cpb* molecular standards were shown to provide positive outcomes in at least 7 and 4 replicates, respectively, which suggests slightly better repeatability of the 18S rRNA-RPA assay than its Cpb counterpart in a low template scenario.

The performance of 18S rRNA-RPA assay was subsequently evaluated for its sensitivity and specificity by assessing archived DNA samples extracted from VL, CL and uninfected control specimens. In addition, pan-*Leishmania* detection capability of our previously developed kDNA-RPA assay was clinically evaluated as well. While both the pan-*Leishmania* RPA assays were able to specifically detect infected samples, in terms of sensitivity, the 18S rRNA-RPA assay (95%) showed superiority over kDNA-RPA assay (60%) in detecting parasite DNA in CL samples that were previously confirmed to be positive in real-time PCR. On the other hand, kDNA-RPA assay was 100% sensitive in detecting parasite DNA in VL and PKDL samples, whereas, poor sensitivity of the 18S rRNA-RPA assay was observed for these cases. This could be pertaining to the low analytical sensitivity of the assay (Supplementary Fig. S6) resulting from lower copy number of the *18S rRNA* gene than the kDNA (~10,000 copies) per genome. Nevertheless, the combinatorial results of the assays signify over 95% sensitivity and absolute specificity against real-time PCR assay for the detection of all types of leishmaniasis. Furthermore, we performed combinatorial assay comprised of the molecular standards of *18S rRNA* and kDNA, and found no inhibition of amplification of either of the targets (not shown). However, further testing on genomic standards will be needed to confirm this result for field implementation. Nevertheless, the RPA assay generates results 6 to 9 times faster than the real-time PCR and requires less equipment and personnel training, thus making it an ideal fit for point-of-need testing in resource-limited settings. In terms of cost-effectiveness, a typical RPA reaction has been reported to cost approximately $2.7 to $4.3 Mondal et al., 2016; Oshiki et al., 2022; Xing et al., 2017, which is expected to go down further. Moreover, isothermal applicability of the assay in the range of 25 to 42 °C has allowed simplification and portability of instruments such as a real-time fluorometer. Depending on integrated facilities and the relative capacity of the number of reactions it can accommodate, the cost of a fluorometer may range between $5000 and $9000, which is, however, several times cheaper than the regular PCR thermocycler which costs around $30,000. For real-time point-of-need diagnosis, the deployment of the RPA assay in the field settings of leishmaniasis-endemic areas has been tested with success in self-contained mobile suitcase laboratories equipped with both nucleic acid extraction and detection systems (Ghosh et al., 2021). A pan-*Leishmania* RPA assay incorporated in a mobile laboratory setup would also be advantageous in combating an outbreak in case a nonendemic species is being suspected in an endemic area or if multiple cohabiting species have similar potential to spread into nearby nonendemic areas (Al-Bajalan et al., 2018). Furthermore, for ecoepidemiology surveys that are important to understand transmission dynamics, a rapid cost-effective way of pan-*Leishmania* screening in a large number of sand fly and potential reservoir samples would leverage the control initiatives in cohabiting endemic zone.

In fact, several pan-*Leishmania* detection approaches have been developed to date which includes both PCR-based and isothermal assays. A SYBR green-based real-time PCR that targets the spliced-leader RNA sequence was shown to have detection limits of less than 0.01 parasite equivalents in a reaction for different source specimens Pareyn et al., 2020; Eberhardt et al., 2018. Another Taqman probe-based real-time PCR assay that targets kDNA was shown to successfully detect parasite DNA in VL and CL-derived specimens with absolute sensitivity and specificity (Wu et al., 2020). However, real-time PCR techniques are still largely confined to enriched laboratories due to its technical and thermal maintenance. Isothermal assays can be an attractive solution to this for both qualitative and quantitative assessment (Kha et al., 2021). Also, a simple colorimetric loop-mediated isothermal amplification (LAMP) technique targeting the *18S rRNA* gene has been previously developed for the rapid detection of a range of *Leishmania* species (Sriworarat et al., 2015). Nevertheless, RPA is advantageous over LAMP as the former offers faster time to result, simpler primer design, longer target sequence, more tolerance to inhibitors, and dispensability of heating source. Our observation in this study shows the prominence of *18S rRNA* encoding chromosomal DNA and kDNA-targeting and fluorescence probe-based RPA assays for use in pan-*Leishmania* detection of genomic DNA from culture as well as clinical samples, with detection capability of less than 1 parasite equivalent limit of molecular DNA standard. However, this study has evaluated the pan-*Leishmania* RPA assays for only the 7 most common infectious *Leishmania* species, whereas *L. mexicana, L. guyanensis, L. peruviana,* and *L. panamensis* also have wide geographical distributions and can cause mucosal, cutaneous and diffuse manifestations (n.d.).

Hence, further validation of single or combinatorial assays should be performed for the other known pathogenic species and with a broad clinical sample size to understand their potentiality for field implementation. Moreover, more data are required regarding the performance of the pan-*Leishmania* RPA assay in other endemic settings in different parts of the world and with multifarious clinical samples. In this regard, low-cost, efficient nucleic acid extraction methods that would suit the developed RPA assays for leishmaniasis with different topical manifestations are desired.

5. Conclusion

The use of a pan-*Leishmania* RPA assay can be advantageous for rapid, sensitive and specific detection of pathogenic *Leishmania* species in suspects, especially, at nonendemic and/or low resource settings and to monitor introduction/transmission of non-endemic species in endemic sites. However, before its recommendation, further validation of the pan-*Leishmania* assays alone or in a combinatorial assay format that is potentially incorporable in a mobile suitcase laboratory through a prospective study is merited.

Authors' contributions

All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing interests.

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Supplementary materials

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