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Parasitology

Identification of Old World *Leishmania* spp. by specific polymerase chain reaction amplification of cysteine proteinase B genes and rapid dipstick detection

Thierry Laurent[a,b], Gert Van der Auwera[a,*], Mallorie Hide[c], Pascal Mertens[b], Wilber Quispe-Tintaya[a,e], Stijn Deborggraeve[a,d], Simonne De Doncker[a], Thierry Leclipteux[b], Anne-Laure Bañuls[c], Philippe Büscher[a], Jean-Claude Dujardin[a]

[a]*Department of Parasitology, Institute of Tropical Medicine Antwerp, 2000 Antwerp, Belgium*
[b]*Coris BioConcept, 5030 Gembloux, Belgium*
[c]*Génétique et Evolution des Maladies Infectieuses, IRD/CNRS (UMR 2724), F-34394 Montpellier, France*
[d]*Laboratory of Medicinal Chemistry, Rega Institute for Medicinal Research, Katholieke Universiteit Leuven, 3000 Leuven, Belgium*
[e]*Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461, USA*

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Abstract

We used the cysteine proteinase B (*cpb*) gene family of the trypanosomatid genus *Leishmania* as a target to develop rapid, specific, and easy-to-use polymerase chain reaction (PCR) tests to discriminate *Leishmania infantum*, *Leishmania donovani*, *Leishmania tropica*, *Leishmania aethiopica*, and *Leishmania major*. Identification of all 5 Old World species and validation of intraspecies variability are features lacking in other species-specific PCRs. Amplicon analysis was done on agarose gels and was further simplified by using an oligochromatography dipstick to detect *L. infantum* and *L. donovani* products. Because the analytical sensitivity is lower than that of certain other species- and genus-specific PCRs, our assays are especially valuable for use on cultured isolates or directly on cryostabilates. As such, they can be implemented by research and health centers having access to culturing, DNA isolation, and PCR.

Keywords: Cysteine protease B; Leishmaniasis; Trypanosomatidae; Oligochromatography; Dipstick; Differential diagnosis

1. Introduction

Leishmaniasis is a vector-borne disease transmitted by sand flies, caused by a protozoan parasite of the genus *Leishmania*. It is endemic in 88 countries on 4 continents, and in the Old World, 5 pathogenic species are encountered: *Leishmania donovani*, *Leishmania infantum*, *Leishmania tropica*, *Leishmania aethiopica*, and *Leishmania major*. Species identification has important practical implications, both for prevention and treatment: First, the mode of transmission can differ, depending on the involved hosts and reservoirs; for example, transmission of *L. donovani* is thought to be anthroponotic, whereas that of *L. infantum* is essentially zoonotic (Lainson and Shaw, 1987). Second, in some cases, sand fly vector specificity is species dependent (Kamhawi, 2006; Volf and Myskova, 2007). Third, the clinical profile differs according to the species; although visceral leishmaniasis (VL) (or kala-azar) is caused by *L. infantum* or *L. donovani*, cutaneous lesions are generally the result of infection with *L. infantum*, *L. tropica*, *L. aethiopica*, or *L. major* (Lainson and Shaw, 1987). Finally, disease progression and treatment outcome are species dependent; for example, *L. tropica* does not respond well to chemotherapy and causes infections of a chronic nature, whereas *L. major* tends to be self-curing (Blum et al., 2004; Pratlong et al., 2004). In the current context of worldwide (re)emergence and spreading of leishmaniasis (Desjeux, 2004), the relevance of species identification further gains importance.

As with many parasites, the taxonomy of the genus *Leishmania* is very complex because species definitions and boundaries are hard to define. Microscopy is of limited use, and currently, the golden standard is multilocus enzyme electrophoresis (MLEE, also known as zymodeme typing)

* Corresponding author. Tel.: +32-32476586; fax: +32-32476359.
E-mail address: gvdauwera@itg.be (G. Van der Auwera).

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(Rioux et al., 1990), which is based upon the relative electrophoretic mobility of selected enzymes. However, MLEE presents some drawbacks: it requires large culture volumes and specialized equipment, it is costly and laborious, and results are hard to compare between laboratories. These restrictions make the technique not generally applicable.

In contrast, polymerase chain reaction (PCR) is a commonly used technique for pathogen detection and typing, and has already been used for *Leishmania* spp. identification with a variety of targets such as *rDNA-ITS1*, *gp*63, *kDNA*, and miniexons. The main concept has been post-PCR analysis by cleavage with restriction enzymes or sequencing (Dujardin et al., 2002; Foulet et al., 2007; Guerbouj et al., 2001; Marfurt et al., 2003a, 2003b; Mauricio et al., 2006; Quispe-Tintaya et al., 2004; Schönian et al., 2003; Spanakos et al., 2008; Zemanová et al., 2007). In addition, a complementary approach has been explored, in which species-specific fragments are amplified (Arora et al., 2008; Eisenberger and Jaffe, 1999; Gramiccia et al., 1992; Haralambous et al., 2008; Hide and Bañuls, 2006; Jirků et al., 2006; Piarroux et al., 1993). However, the species-specific PCR assays reported so far do not allow to identify all species and amplify different DNA targets (Table 1).

In the present study, we developed 5 species-specific PCR tests that can discriminate each of the Old World species: *L. infantum*, *L. donovani*, *L. tropica*, *L. aethiopica*, and *L. major* (further abbreviated as LI-, LD-, LT-, LA-, and LM-PCR, respectively) in cultured parasite isolates. They are all based on the species-specific amplification of the same target, that is, the gene encoding cathepsin L-like cysteine proteinase B (*cpb*), a major antigen of *Leishmania* parasites. In addition, we established the proof of principle of the adaptation of 2 of these assays (LI- and LD-PCR) for oligochromatography (OC) detection, a rapid dipstick test for visualization of specific amplified products (Akinwale et al., 2008; Claes et al., 2007; Deborggraeve et al., 2006, 2008; Dineva et al., 2005; Edvinsson et al., 2004).

2. Materials and methods

2.1. Samples

An overview of all strains used is presented in Table 2. *L. tropica*, *L. aethiopica*, and *L. major* DNA was provided by J. P. Dedet (University of Montpellier, France) and G. Schönian (Institut für Mikrobiologie und Hygiene, Berlin, Germany). DNA of *L. infantum* and *L. donovani* strains was provided by J.P. Dedet and I. Mauricio (London School of Hygiene and Tropical Medicine, London, UK). Based on zymodeme typing (Rioux et al., 1990) and molecular methods (Mauricio et al., 2001; Quispe-Tintaya et al., 2004, 2005; Zemanová et al., 2004), some discrepancies were found in discriminating the latter 2 species, which together make up the *L. donovani* complex. For the purpose

Table 1
Overview of Old World *Leishmania* species-specific PCR-agarose gel assays

Reference	Target[a]	Positive identification[b]	Missing validation[c]	Principle[d]	PCRs needed[e]
Smyth et al. (1992)	kDNA	D + I + M	A	SD	1
Bhattacharyya et al. (1993)	kDNA	M/T	A	SA + RAPD	2
Meredith et al. (1993)	kDNA	D/M/T/A	I	SA	4
Meredith et al. (1993)	*ssu-rDNA*	D/I		SA	1
Piarroux et al. (1993)	Repetitive DNA	D + I + M/A		SA + SD	1
Pogue et al. (1995)	Unknown	D/I/M/T	A	RAPD	4
Ravel et al. (1995)	kDNA	D + I/M	T/A	SA + SD	1
Schönian et al. (1996)	Unknown	D + I/M/T	A	RAPD	3
Eisenberger and Jaffe (1999)	Unknown	D + I/M/T/A		PPIP-PCR	1
Aransay et al. (2000)	kDNA	D + I + T/M + A		SD	1
Hanafi et al. (2001)	Unknown	D + I/M/T	A	RAPD	1
Hanafi et al. (2001)	RAPD target	D + I		SA	1
Salotra et al. (2001)	kDNA	D + I	A	SA	1
Anders et al. (2002)	kDNA	D + I + T/M/A		SD	1
Mahboudi et al. (2002)	kDNA	D/I/M	T/A	SD	1
Hide and Bañuls (2006)	*cpb*	D/I	A	SA + SD	1
Jirků et al. (2006)	Unknown	T		SA	1
Arora et al. (2008)	*hsp70*	D		SA	1
Haralambous et al. (2008)	*k26/haspB*	D/I	A	SA + SD	1
This article	*cpb*	D/I/M/T/A		SA	5

kDNA = kinetoplast DNA (minicircles); *ssu-rDNA* = small subunit ribosomal DNA; RAPD = random amplification of polymorphic DNA; *hsp70* = heat-shock protein 70; *k26/haspB* = hydrophilic acylated surface protein B; D = *L. donovani*; I = *L. infantum*; M = *L. major*; T = *L. tropica*; A = *L. aethiopica*; SA = species-specific amplification; SD = size discrimination; PPIP-PCR = permissively primed intergenic polymorphic PCR.

[a] Molecular target of the assay.

[b] Species that can be positively identified using the assay. A joining "+" sign indicates species that cannot be discriminated, whereas a "/" sign indicates the different groups that are distinguished in each assay.

[c] Species that were not validated in the respective publication.

[d] Principle upon which the assay is based.

[e] Number of PCRs needed for a positive identification of all distinguishable groups.

Table 2
Results from species-specific PCRs analyzed on agarose gel and by OC[a]

Strain (international WHO code)	Species	PCR-agarose gel					PCR-OC	
		LD	LI	LA	LM	LT	LD	LI
MHOM/IN/-/DEVI	*L. donovani*	+	−	−	−	−	+	−
MHOM/IN/96/THAK35		+	−	−	−	−	+	−
MHOM/IN/54/SC23		+	−	−	−	−	+	−
MHOM/ET/-/HUSSEN		+	−	−	−	−	+	−
MCAN/SD/00/LEM3946		+	−	−	−	−	+	−
MHOM/SD/97/LEM3429[b]		+	−	−	−	−	+	−
MHOM/SD/97/LEM3463[b]		+	−	−	−	−	+	−
MHOM/ET/72/GEBRE1[b]		+	−	−	−	−	+	−
MHOM/SD/82/GILANI		+	−	−	−	−	+	−
MHOM/SD/62/3S[c]		+	−	−	−	−	+	−
MHOM/SD/97/LEM3472[c]		+	−	−	−	−	+	−
MHOM/FR/78/LEM75	*L. infantum*	−	+	−	−	−	−	+
MHOM/FR/95/LPN114		−	+	−	−	−	−	+
MHOM/ES/93/PM1		−	+	−	−	−	−	+
MHOM/FR/97/LSL29		−	+	−	−	−	−	+
MHOM/ES/86/BCN16		−	+	−	−	−	−	+
MHOM/PT/00/IMT260		−	+	−	−	−	−	+
MHOM/FR/80/LEM189		−	+	−	−	−	−	+
MHOM/ES/91/LEM2298		−	+	−	−	−	−	+
MHOM/IT/93/ISS800		−	+	−	−	−	−	+
MHOM/ES/88/LLM175		−	+	−	−	−	−	+
MHOM/ES/92/LLM373		−	+	−	−	−	−	+
MHOM/IT/94/ISS1036		−	+	−	−	−	−	+
MHOM/FR/96/LEM3249		−	+	−	−	−	−	+
MHOM/MT/85/BUCK		−	+	−	−	−	−	+
MHOM/ET/81/1123-81	*L. aethiopica*	−	−	+	−	−	−	−
MHOM/ET/83/32-83		−	−	+	−	−	−	−
MHOM/ET/80/1561-80		−	−	+	−	−	−	−
MHOM/ET/94/Abauy		+	−	+	−	−	+	−
MHOM/ET/94/1769		−	−	+	−	−	−	−
MHOM/ET/94/1470		−	−	+	−	−	−	−
MHOM/ET/94/Gere		+	−	+	−	−	+	−
MHOM/ET/94/Wandera		+	−	+	−	−	+	−
MHOM/ET/87/Kassaye		−	−	+	−	−	−	−
MPSM/SA/89/SABIR-1	*L. major*	−	−	−	+	−	−	−
MHOM/TN/97/LPN162		−	−	−	+	−	−	−
MHOM/SU/73/5ASKHT		−	−	−	+	−	−	−
MHOM/BF/96/LIPA538		−	−	−	+	−	−	−
MHOM/UZ/02/17 h		−	−	−	+	−	−	−
MHOM/UZ/98/2M		−	−	−	+	−	−	−
MHOM/UZ/98/IsvM-08 h		−	−	−	+	−	−	−
MHOM/IL/03/LRC-L962		−	−	−	+	−	−	−
MHOM/IQ/66/L75	*L. tropica*	−	−	−	−	+	−	−
MHOM/PS/01/ISL593		−	−	−	−	+	−	−
MHOM/PS/01/ISL590		−	−	−	−	+	−	−
MHOM/PS/01/ISL595		−	−	−	−	+	−	−
MHOM/IL/01/LRC-L838		−	−	−	−	+	−	−
IARA/IL/02/LRC-L906		−	−	−	−	+	−	−
MHOM/PS/03/ISLAH721		−	−	−	−	+	−	−
MHOM/IL/02/LRC-L863		−	−	−	−	+	−	−
MHOM/PS/03/152JnF32		−	−	−	−	+	−	−
MHOM/PS/03/LRC-L1022		−	−	−	−	+	−	−

WHO = World Health Organization.

[a] A positive result (i.e., an amplicon of the correct size or a corresponding line on the OC dipstick) is indicated by "+". All other cases (i.e., negative results) are indicated by "−".

[b] Typed as *Leishmania archibaldi* by MLEE analysis (Lukeš et al., 2007).

[c] Typed as *L. infantum* by MLEE analysis (Lukeš et al., 2007).

of this study, classification was based on Lukeš et al. (2007), which is by far the most comprehensive analysis to date.

The detection threshold of our methods was defined by analysis of 180 μL of healthy human blood seeded with an amount of promastigotes ranging from 10^6 down to 1, and 3 nonseeded blood samples were used as negative controls. DNA was extracted with the QIAamp DNA Mini Kit using buffers AS1 and AS2 (http://www.qiagen.com, Qiagen, Venlo, The Netherlands) and eluted in 50 μL. The 2 strains we used are *L. donovani* MHOM/SD/-/1-S and *L. infantum* MHOM/MA/67/ITMAP263. Using the same kit but an elution volume of 100 μL, we also extracted DNA directly from 48 cryostabilates in DMSO (H. Louzir, Institut Pasteur de Tunis, Tunis-Belvédère, Tunisia).

2.2. Sequences

Sequences of *cpb* genes from all 5 target species were retrieved from the EBI database (http://www.ebi.ac.uk). In addition, annotated cathepsin L-like proteases of *L. major* strain Friedlin from the GeneDB database (http://www.genedb.org) were included in the data set. Although, currently, a complete coding *cpb* sequence is available for both *L. aethiopica* and *L. tropica* (Kuru et al., 2007), at the start of this study, no data were known. Therefore, we determined partial *cpb* sequences from strains *L. aethiopica* MHOM/ET/72/L100 and *L. tropica* MHOM/SU/74/K27. These were PCR amplified with sense primer cpbFor2 and antisense primer cpbRev2 or cpbRev4 (Table 3), all of which were designed in a conserved part of *cpb*. We used Silverstar *Taq* DNA polymerase (Eurogentec, Seraing, Belgium) with 0.83 mmol/L $MgCl_2$ and 25 PCR cycles to minimize errors due to the lack of proofreading activity. The obtained amplicons were cloned in vector pCR2.1 with the TOPO-TA cloning kit (Invitrogen, La Jolla, CA), according to the manufacturer's instructions. Eleven clones from each amplicon were sequenced.

2.3. Species-specific PCR amplifications

Table 3 lists all primers (http://www.sigmaaldrich.com, Sigma-Aldrich/Genosys, Bornem, Belgium) that were used in the species-specific amplifications, and occasionally, mismatches were introduced to prevent cross-reactions to nontarget species. Each primer pair amplifies only one of the different *cpb* copies present in a particular species (Fig. 1). All reactions were performed in an iCycler machine (BioRad, Hercules, CA), using thin-walled PCR tubes of 0.2 mL (BioRad). Concentrations of genomic DNA extracted from promastigote cultures were measured spectrophotometrically. Polymerase chain reaction amplicons were detected by analyzing 5 or 10 μL of the PCR mix on an ethidium bromide–stained 2.5% agarose gel. The PCR was scored positive only when an amplicon of the expected size (Fig. 1) was observed.

The LD- and LI-PCRs were performed in 30 μL containing 1.5 U HotStarTaq DNA polymerase (Qiagen) and the accompanying 1× HotStarTaq buffer (including 1.5 mmol/L $MgCl_2$), 0.2 mmol/L of each deoxynucleoside triphosphate, 0.33 μmol/L of each primer (Table 3), and 20 ng of promastigote DNA or 5 μL of extracted DNA from seeded blood samples. Cycling was done as follows: initial denaturation for 15 min at 95 °C, 40 cycles consisting of 30 s at 95 °C, 15 s at 59 °C, 15 s at 72 °C, and, finally, an extra elongation

Table 3
Oligonucleotides

Name	Sequence (5′→3′)[a]	Function	Specificity[b]
Species-specific PCR primers			
infcpbE	GTCTTACCAGAGCGGAGTGCTACT	S	*L. infantum*
Inf2.1	ATAACCAGCCATTCGGTTTTG	AS	*L. infantum*
cpbF2.1	GCGGCGTGATGACCAGC	S	*L. donovani*
Do2.1	CAATAACCAGCCATTCGTTTTTA	AS	*L. donovani*
MATRAE2	GGCGATGGTGGAGCAGATGATCT	S	*L. major* *L. tropica* *L. aethiopica*
Ma4.1	CGGTTCTCGTAGCACACTTGTTG	AS	*L. major*
Tr4.1	CTCCCCCGTTCGGAT	AS	*L. tropica*
Ae2.1	AGTACGTGCACATCAGCACATGGG	AS	*L. aethiopica*
Gold-labeled detection probe			
DoInf2	TCGAGTTCTTGATCACCCAGTACG	OC	*L. donovani* *L. infantum*
PCR primers for amplification of sequenced fragments			
cpbFor2	ACGCCAGCTCCTTCATGTC	S	*Leishmania*
cpbRev2	CATAGAGGAGCCGCCTCCCC	AS	*Leishmania*
cpbRev4	GCAGGTGTTCTTGCTCGAGCC	AS	*Leishmania*

S: sense sequence; AS: antisense sequence; OC: gold-labeled OC detection probe (conjugate).

[a] To prevent cross-reactions, self-priming, primer-dimers, or internal secondary structures, some residues do not match the target sequence.

[b] See also Fig. 1 for an overview of the targeted *cpb* copies.

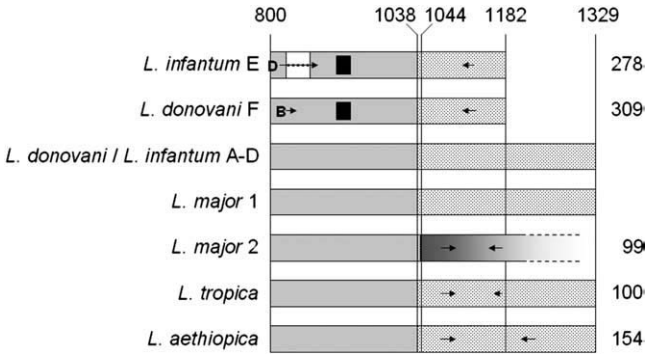

Fig. 1. Schematic overview of the *cpb* types and PCR amplicons in the 5 target species, drawn to scale. Nucleotide positions relative to the start of the coding sequence are indicated on top. *L. infantum*, *L. donovani*, and *L. major* strains harbor 2 different size versions of the protein, whereas in *L. tropica* and *L. aethiopica*, only 1 size has been found. The assays described in this study target only 1 of the variants in each species, but the others are shown for clarity. Apart from a 39-bp deletion in *cpbE* (white block), no length variation and only moderate sequence variation occurs in the 1st part of the coding sequence (shown in light gray starting from nucleotide 800). Several length variants exist of the 3′ end of the coding sequence (speckled), but in each species, the 1329-bp (443 AA) variant is present. Only the light gray regions are aligned, and the *cpbE* deletion was not considered to indicate the nucleotide positions on top, that is, 39 bp need to be subtracted from the indicators downstream of the deletion to obtain the correct position in *L. infantum cpbE*. The annealing position of sense and antisense PCR primers (Table 3) are indicated, and obtained amplicon lengths are shown on the right. It should be noted that the species-specific primers for *L. major* amplify a noncoding sequence downstream of the protein coding sequence (shown in fading dark gray). For OC detection, the labels attached at the 5′ end of the primers are indicated by B (biotin) and D (DIG), and the annealing position of the gold-labeled antisense detection probe DoInf2 (Table 3) is represented by the black rectangle.

step of 5 min at 72 °C. When detection of the PCR amplicons was done by OC, the sense primers were labeled and used at a concentration of 1.33 μmol/L. In such case, primer cpbF2.1 (Sigma-Aldrich/Genosys) contained a 5′ biotin and primer infcpbE (Eurogentec) a 5′ digoxigenin (DIG) moiety. Consequently, *L. donovani*–specific amplicons are labeled with biotin and *L. infantum* products with DIG. In addition, 1.2 U HotStarTaq DNA polymerase was used, and the final elongation step was followed by 1 min of denaturation at 95 °C and rapid (snap) cooling to 4 °C before OC.

Species-specific LM-, LT-, and LA-PCRs were run in 25 μL containing 1× Silverstar Taq buffer and 0.8 U Silverstar Taq (Eurogentec), 1 mmol/L $MgCl_2$, 0.2 mmol/L of each dNTP, 0.33 μmol/L of each primer (Table 3), and 20 ng DNA. Cycling was done as follows: initial denaturation for 5 min at 95 °C, 35 cycles consisting of 30 s at 95 °C, 30 s at 57 °C, 30 s at 72 °C; and, finally, an extra elongation step of 5 min at 72 °C. LM- and LT-PCRs on 1 μL of cryostabilate DNA were done in 30 μL with 1 U polymerase, using 35 and 40 cycles, respectively, with the rest of the conditions unchanged.

2.4. Oligochromatography

The principle of OC detection of PCR amplicons is illustrated in Fig. 2, and primer and probe positions relative to *L. infantum* and *L. donovani* sequences are shown in Fig. 1. It should be noted that the specificity of the species-specific assays is determined at the PCR level by the amplification of sequences from only one of the species. In addition, the gold-labeled detection oligonucleotide DoInf2 (Table 3) is complementary to an internal amplicon sequence, thereby ensuring that aspecific amplification products formed during the reaction (such as primer–dimers or human amplicons) remain undetected. Several measures are taken to ensure specific and efficient hybridization of DoInf2: 1) the migration mix containing the amplicons is kept at 55 °C before and during the OC detection to prevent aspecific hybridization; 2) the biotin- or DIG-labeled PCR primer is used at a concentration 4 times that of the other primer to result in more single-stranded template available for hybridization; and 3) before OC detection, PCR mixes are denatured and snap cooled to prevent formation of large stable duplexes that do not separate at the temperature used to

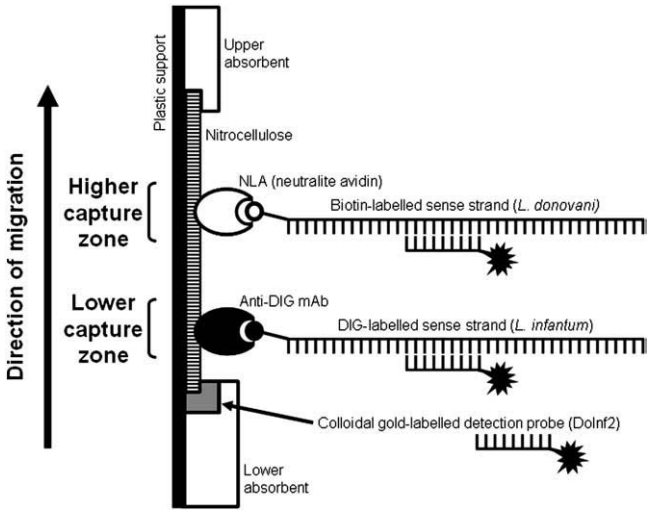

Fig. 2. Schematic side view of the OC dipstick and detection principle. The strip (Coris BioConcept) is composed of a plastic support laminated with an upper and lower absorbent, and a nitrocellulose membrane (hatched) containing 2 coated areas, further referred to as lower and higher capture zones. The lower absorbent contains the colloidal gold-labeled oligonucleotide detection probe DoInf2 (Table 3), which hybridizes to the sense strand of the *cpbE* (DIG-labeled) and *cpbF* (biotin-labeled) amplicons from *L. infantum* and *L. donovani*, respectively. When the OC strip is dipped in a solution containing PCR products from either species, the amplicons start migrating up the strip, first passing through the lower absorbent where they hybridize to the gold-labeled DoInf2 probe (conjugate). This gold-labeled complex continues to migrate in the nitrocellulose, first reaching the lower capture zone coated with anti-DIG monoclonal antibody (Biogenesis, Poole, UK). If the PCR products are DIG labeled, which is the case only for *L. infantum cpbE* amplicons, the complex will be captured at this site. This causes an accumulation of gold particles, which is visible to the naked eye as a red line. Uncaptured complexes migrate further up to reach the higher capture zone consisting of an NLA coating. *L. donovani cpbF* amplicons bind to this region because they are biotin labeled, leading to an accumulation of gold particles at the higher capture zone. In case neither DIG- nor biotin-labeled amplicons are present or in case they represent aspecific amplification products that do not hybridize to the gold-labeled detection probe, no accumulation of gold particles occurs, and both capture zones remain colorless.

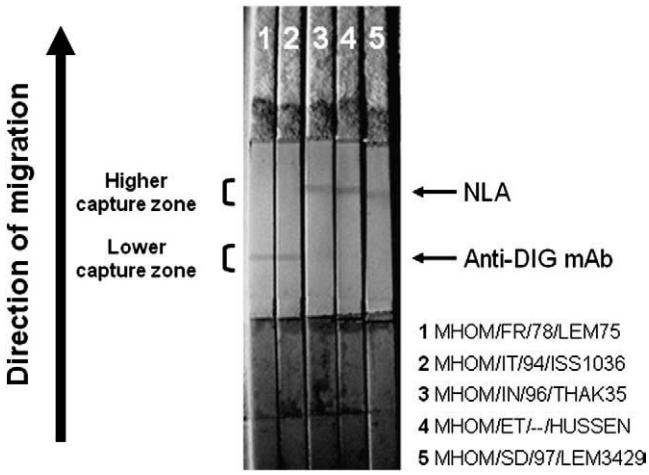

Fig. 3. Oligochromatography (OC) result from pooled detection using 5 characterized reference strains. Strains are indicated by lane numbers, whereby lanes 1 and 2 represent *L. infantum* strains, and lanes 3 to 5 represent *L. donovani* strains (Table 2). Species-specific LD- and LI-PCRs were performed separately, and 10 μL from each were pooled for OC detection. The visualization of accumulated gold particles in the lower and higher capture zones is indicated by arrows.

run the OC. In contrast to agarose gels, in which the specificity of the reaction is confirmed only by length discrimination, OC specificity is governed by specific internal gold labeling and separated detection of differently labeled amplicons. Dipsticks for OC detection were manufactured by Coris BioConcept, Gembloux, Belgium (http://www.corisbioconcept.com) on demand, and the production process includes a quality control system to minimize batch-to-batch variation and to ensure the quality of materials.

Two protocols were followed for the PCR product detection by OC: single and pooled detection. In the single detection, LI- and LD-PCRs performed on a particular sample were analyzed on separate dipsticks. To this end, 20 μL of the denatured and snap-cooled (see above) PCR reaction was mixed with an equal volume of OC migration buffer (SUL-3702, Coris BioConcept) preheated at 55 °C in a water bath, and the OC strip (Coris BioConcept) was dipped immediately in this mix. Migration of the PCR amplicons was carried out for 5 to 10 min while keeping the mix at 55 °C; after which, the presence of amplicons was determined by visual inspection of the capture zones (Fig. 2). The same procedure was followed for the pooled detection (Fig. 3), except that 10 μL from both the LD- and LI-PCRs were mixed with 20 μL of OC migration buffer for analysis on a single dipstick, allowing detection of both amplicons. In both procedures, appearance of the excess conjugate as a red smear on the upper absorbent indicates that the migration occurred properly.

3. Results

3.1. Analysis of cpb sequences

Four different sequences were identified in 11 cloned *cpb* PCR amplicons from strain *L. tropica* MHOM/SU/74/K27, which were deposited at the EBI and GenBank sequence databases with accession numbers DQ373069, DQ373070, DQ373071, and DQ373072. In 11 cloned amplicons from *L. aethiopica* MHOM/ET/72/L100, 3 different sequences were found, and these were given accession numbers DQ373073, DQ373074, and DQ373075.

The available *cpb* sequences of the Old World *Leishmania* spp. were aligned, and a schematic representation is shown in Fig. 1. Apart from a 39-nucleotide (13 amino acids) deletion in the *cpbE* copy of *L. infantum*, no length variation and only minor sequence variation is present among the first 1038 nucleotides or 346 amino acids of the coding sequences of all species (Fig. 1). The remaining part of the coding sequence displays considerable length and sequence variation. The 1329-nucleotide (443 amino acids) variant is encountered in strains from each species, but in *L. donovani*, *L. major*, and *L. infantum*, an additional shorter *cpb* variant was found (Hide and Bañuls, 2008; Hide et al., 2007; Mundodi et al., 2002).

3.2. Species-specific PCRs

All 5 species-specific PCRs were performed on 52 DNAs isolated from promastigote cultures (Table 2). A species-specific amplicon of the correct size (Fig. 1) was obtained in all 52 cases, when using 20 ng of cultured promastigote DNA as PCR template. For 3 *L. aethiopica* strains, however, an additional *L. donovani*-specific amplicon was obtained, whereas no cross-reacting products were amplified in the remaining 49 PCRs. Apart from detection on agarose gels, the LD- and LI-PCRs were analyzed by single detection OC using a slightly modified PCR protocol (see Materials and methods, results listed in Table 2). The results were identical as for the agarose gel analyzed reactions, including the cross-reaction observed for the 3 *L. aethiopica* strains. All PCRs remained negative when tested on 3 healthy human blood samples.

The fact that the 3 *L. aethiopica* strains, MHOM/ET/94/Gere, MHOM/ET/94/Wandera, and MHOM/ET/94/Abauy, all showed cross-reaction in the LD-PCRs when analyzed either on agarose gel or OC strongly indicated that genuine *L. donovani cpbF* amplicons are amplified. Indeed, whereas specificity in agarose gels is based on size, it is governed by the internal gold-labeled oligonucleotide hybridization sequence in OC, and hence, detection with both methods can hardly be a coincidence. As expected, sequencing showed the LD-PCR amplicons obtained from these *L. aethiopica* strains to be identical to the *cpbF* gene of genuine *L. donovani*. To exclude the possibility of an *L. donovani* contamination in our cultures or DNA, we obtained new stocks of these strains (J.P. Dedet, University of Montpellier) and tested anew, but results were the same. When analyzing the ribosomal internal transcribed spacer 1 (*rDNA-ITS1*) with 3 different newly developed primer combinations specific for the *L. donovani* complex, an *L. donovani*-specific amplicon was obtained for all 3 strains (results not shown). This agrees with the *rDNA-ITS1* RFLP analysis of MHOM/ET/94/Gere,

which shows a mix of *L. aethiopica* and *L. donovani* fragments (Fig. 2A in Schönian et al., 2003).

The analytical sensitivity of the PCR-OC assays was tested by analyzing DNA isolated from blood spiked with a known amount of promastigotes, using single detection OC. For both the LD- and LI-PCR assays, the detection limit was 14 promastigotes/μL of blood. Contrary to the LD-PCRs, detection of LI-PCR amplicons on agarose gel was up to 100 times more sensitive than detection on OC dipstick. Pooled detection OC was tested on DNA from 5 characterized promastigote cultures (Fig. 3). The 2 *L. infantum* and 3 *L. donovani* strains were correctly identified by OC because only their respective capture region was marked with an accumulation of gold beads. Of 48 DNAs isolated from cryostabilates, 47 tested positive in the LM-PCR and 1 tested positive in the LT-PCR, using agarose gel detection.

4. Discussion

Five species-specific PCRs targeting the Old World *Leishmania cpb* gene were developed, permitting species identification of *L. donovani*, *L. infantum*, *L. tropica*, *L. major*, and *L. aethiopica*. In each PCR, only one of the *cpb* types present in the respective species is amplified (Fig. 1). When adopting the species boundaries between *L. donovani* and *L. infantum* as proposed in Lukeš et al. (2007), all PCRs proved to be 100% specific and sensitive when tested on negative human control DNA and 20 ng of culture DNA of the 5 target species (Table 2).

The above figures ignore the *L. donovani*-specific amplicons obtained from 3 *L. aethiopica* strains in the LD-PCR because they do not result from a cross-reaction of the specific *cpb* assay. Indeed, because *L. donovani*-specific *rDNA-ITS1* amplicons are amplified from these DNAs as well, 2 hypotheses can be considered: either the original culture is a mix of both species or these strains represent an *L. aethiopica*/*L. donovani* hybrid. Natural hybrids have already been described (e.g., Ravel et al., 2006) and would, in this case, be promoted by the fact that both species are sympatric in parts of Eastern Africa. Further elaboration on this matter lies outside the scope of the present article.

Compared with other assays based on PCR amplification immediately followed by agarose gel detection (Table 1), our test has the advantage that it is universally applicable in the Old World. All 5 species can be identified, and all PCRs were validated against a panel of at least 8 isolates per species, thereby considering inter- as well as intraspecies variation. None of the other assays listed in Table 1 distinguishes all 5 Old World species, which limits their use to particular regions with preexisting knowledge of species distribution. In addition, the specificity of most tests was not validated against all species, and especially the nontarget intraspecies variability was poorly documented because only 1 strain was generally included in the analysis. This entails the danger of newly introduced species going undetected because they could be erroneously typed. Finally, tests based on random amplification of polymorphic DNA and permissively primed intergenic polymorphic fingerprints are difficult to interpret and hard to standardize across different laboratories. There are, however, also downsides to the approach presented in this article. First, its sensitivity is generally lower than that of the other assays mentioned in Table 1. In addition, 5 PCRs have to be performed in parallel to identify all species.

We demonstrated the proof of principle of the adaptation of the LI- and LD-PCR assays to an oligochromatographic detection format. This system has several advantages over conventional agarose gels: 1) PCR results can be assessed within 10 min; 2) it is a straightforward technique that requires only a water bath for migration at 55 °C; 3) it does not require toxic DNA intercalating dyes; and 4) it is suitable for standardization and batch-to-batch quality control. By using different capture systems, simultaneous detection of several equally sized amplicons is also possible, as demonstrated in the so-called pooled detection OC of LD-PCR and LI-PCR amplicons: in each of the 5 cultured promastigote DNAs, the correct species was identified (Fig. 3). However, it seemed that the capture method of the PCR amplicons contributes considerably to the overall sensitivity of the method, as illustrated by the fact that contrary to the LD-PCRs, for the LI-PCRs, detection via OC was less sensitive than agarose gel analysis. Labeling primer infcpbE with biotin instead of DIG eliminated this phenomenon (results not shown), indicating that the biotin/Neutralite avidin (NLA) (Belovo, Bastogne, Belgium) capture system is superior to the DIG/anti-DIG system. Hence, currently, pooled detection OC needs further optimization, and the use of a separate OC dipstick for each PCR is recommended.

Overall, the analytical sensitivity of the PCR-OC assays was found to be 14 promastigotes/μL of spiked blood. This is much lower than the 0.005 parasites/μL of blood detectable by similar PCR-OC assays, developed for the diagnosis of *Trypanosoma brucei* (Deborggraeve et al., 2006) and the genus *Leishmania* (Deborggraeve et al., 2008). This may be in part explained by the fact that the latter assays target multicopy ribosomal RNA genes, whereas our assays amplify single-copy *cpbE* and *cpbF* genes (Mundodi et al., 2002). Therefore, future development toward improved sensitivity could be guided by targeting repeated genes showing species-specific DNA polymorphisms, such as *rDNA-ITS1* (Schönian et al., 2003). Alternatively, the OC detection system itself could be improved by modification of the capture method or the use of a more sensitive visualization than the gold bead accumulation detected by the naked eye. The use of nested PCR is another option.

Considering the relatively low analytical sensitivity of our assays, their domain of application is currently essentially limited to typing cultured parasites, although small culture volumes are sufficient. As such, they provide a simple alternative to MLEE, but not to parasite detection. Species

determination can be done on cultures and, as we have shown, even on DNA extracted directly from DMSO cryostabilates, thereby providing a rapid quality control test for cryobanks. However, as a matter of exploration, we also applied our assays on 12 blood samples of patients with VL from Bakool, Somalia (Raguenaud et al., 2007), 2 bone marrow samples from Nepalese patients with VL, 4 spleen aspirates from canine leishmaniasis, and 50 biopsies from Tunisian patients with cutaneous leishmaniasis (results not shown). In total, only 8 from these 68 samples could be typed and showed the expected species: *L. donovani* in the patients with VL, *L. infantum* in dogs, and *L. major* in the cutaneous lesion (Lainson and Shaw, 1987). The low success rate in these samples illustrates the current lack of sensitivity for use with clinical samples. Nevertheless, this does not decrease the epidemiologically informative value of the successful identifications on a subset of samples.

In conclusion, we describe 5 species-specific PCRs, each of which amplifies a *cpb* fragment only encountered in 1 of the 5 Old World *Leishmania* spp.: *L. donovani*, *L. infantum*, *L. tropica*, *L. major*, and *L. aethiopica*. The presence or absence of a specific amplicon can be assessed on agarose gel or, in the case of *L. donovani* and *L. infantum*, on an OC dipstick. Because our methods are insufficiently sensitive to analyze directly clinical samples, they are especially suitable for typing small parasite cultures or cryostabilates and, thereby, for implementation in health and research centers disposing of culture, DNA isolation, and PCR facilities. Although *cpb* PCR-OC has presently been developed for 2 species only, the species-specific PCRs followed by detection on agarose gel are widely applicable and can be used both for high- and low-throughput applications.

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