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Identification of *Leishmania* by Matrix-Assisted Laser Desorption Ionization–Time of Flight (MALDI-TOF) Mass Spectrometry Using a Free Web-Based Application and a Dedicated Mass-Spectral Library

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ABSTRACT Human leishmaniases are widespread diseases with different clinical forms caused by about 20 species within the *Leishmania* genus. *Leishmania* species identification is relevant for therapeutic management and prognosis, especially for cutaneous and mucocutaneous forms. Several methods are available to identify *Leishmania* species from culture, but they have not been standardized for the majority of the currently described species, with the exception of multilocus enzyme electrophoresis. Moreover, these techniques are expensive, time-consuming, and not available in all laboratories. Within the last decade, mass spectrometry (MS) has been adapted for the identification of microorganisms, including *Leishmania*. However, no commercial reference mass-spectral database is available. In this study, a reference mass-spectral library (MSL) for *Leishmania* isolates, accessible through a free Web-based application (mass-spectral identification [MSI]), was constructed and tested. It includes mass-spectral data for 33 different *Leishmania* species, including species that infect humans, animals, and phlebotomine vectors. Four laboratories on two continents evaluated the performance of MSI using 268 samples, 231 of which were *Leishmania* strains. All *Leishmania* strains, but one, were correctly identified at least to the complex level. A risk of species misidentification within the *Leishmania donovani*, *L. guyanensis*, and *L. braziliensis* complexes was observed, as previously reported for other techniques. The tested application was reliable, with identification results being comparable to those obtained with reference methods but with a more favorable cost-efficiency ratio. This free online identification system relies on a scalable database and can be implemented directly in users' computers.

KEYWORDS *Leishmania*, MALDI-TOF, database, identification online

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Leishmaniasis is caused by protozoan parasites that belong to the genus *Leishmania* and are transmitted by the bite of female phlebotomine sand flies. Leishmaniasis is a widespread disease, with 350 million people at risk and 12 million new cases per year in more than 98 countries (1). Currently, 53 *Leishmania* species have been described and are included in the two main phylogenetic lineages, euleishmania and paraleishmania. Among them, 31 species are involved in mammalian parasitism, and 20 species are associated with human leishmaniasis (2). Different clinical forms are commonly observed, including cutaneous, mucocutaneous, and visceral forms. Cutaneous leishmaniasis (CL) affects 1.5 million patients per year and displays a large spectrum of clinical forms, from small self-resolving papules to destructive mucosal lesions. The clinical presentation of CL depends on the *Leishmania* species, but the lesion characteristics and the probable place of contamination are not specific enough to determine the species involved (3). Nevertheless, the patient's travel history is a very important piece of information and could be considered a quality control for diagnostic results. Infections by two species of the *Leishmania* subgenus (*Leishmania major* and *L. mexicana*) show frequent spontaneous cures within a few months (3). On the other hand, the two main species of the *Viannia* subgenus (*Leishmania braziliensis* and *L. panamensis-L. guyanensis*) are associated with a 1 to 15% risk of delayed mucosal metastasis (4). Therefore, *Leishmania* species identification is especially relevant in CL and mucocutaneous leishmaniasis for therapeutic management (5). Moreover, *Leishmania* species identification remains fundamental for understanding complex epidemiological cycles and for setting up disease control measures. Several methods for *Leishmania* identification to the species level are available (reviewed in reference 6). Molecular methods allow *Leishmania* typing directly from clinical samples (e.g., skin biopsy specimens) or from parasite cultures (7). Multilocus enzyme electrophoresis (MLEE), which is based on parasite culture isolation, is considered by the World Health Organization to be the gold standard for parasite typing and is the only technique that has been evaluated for almost all currently identified *Leishmania* species (8). Nevertheless, this method is cumbersome, time-consuming, and used in only a few laboratories worldwide. Alternatively, PCR-based methods, such as restriction fragment length polymorphism (RFLP) or amplicon sequence analysis, have been proposed as methods to identify *Leishmania* species. Although they are simpler than MLEE, they remain time-consuming and are not available everywhere.

During the last decade, mass spectrometry (MS) has been adapted to microorganism identification. Nowadays, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS is integrated into the workflow of many routine laboratories, thus greatly simplifying pathogen identification and improving patient care (reviewed in reference 9). A few studies support the proof-of-concept for *Leishmania* species identification with this method (10–12). However, those studies were done by using in-house databases that are not available to other people working on leishmaniasis. As no reference mass-spectral library (MSL) for *Leishmania* is commercially available, the aim of this study was to construct and test the reliability of a reference MSL using samples from an international collection of *Leishmania* isolates in Montpellier, France (Centre de Ressources Biologiques des *Leishmania* [CRB-Leish]). This library is available through a free online mass-spectral identification (MSI) application.

(This study was presented in part as a poster at the 6th World Congress on Leishmaniasis, Toledo, Spain, 16 to 20 May 2017 [13].)

RESULTS

Construction and testing of the mass-spectral reference database (MSL). The *Leishmania* MSL was built by using the mass spectra of 121 well-characterized *Leishmania* strains from the CRB-Leish collection (Table 1). The MSL was implemented in a specific Web-based application designed for mass-spectral identification (MSI application; https://biological-mass-spectrometry-identification.com/msi/) and then tested with an independent panel of 268 samples (231 *Leishmania* isolates and 37 outgroup controls) from four different laboratories (see Table 2 for a complete description of

TABLE 1 Isolates used for mass-spectral library construction[a]

Section	Subgenus	Complex	Taxon (no. of isolates)	Isolate(s) (WHO code) used for MSL construction
Euleishmania	*L. enriettii* complex	*L. enriettii*	*L. enriettii* (2)	MCAV/BR/45/L88, MCAV/BR/95/CUR2
			L. martiniquensis (3)	MHOM/GP/2008/LEM5748, MHOM/MQ/92/MAR1, MHOM/MQ/94/LEM2870CL
			L. siamensis (1)	MHOM/TH/2010/TR
	Leishmania	*L. donovani*	*L. archibaldi* (8)	MHOM/ET/72/GEBRE1, MCAN/SD/98/LEM3556, MCAN/SD/99/LEM3796, MHOM/TR/2001/EP59, MHOM/SD/97/LEM3463, MHOM/SD/97/LEM3429, MHOM/SD/2002/MW116, MHOM/SD/2002/MW101
			L. donovani (6)	MHOM/IN/00/DEVI, MHOM/IN/80/DD8, MHOM/IN/96/THAK57, MHOM/IN/99/8998, MHOM/TR/2012/LPS55, MHOM/LK/2010/OVN3
			L. infantum (7)	MHOM/EG/87/RTC2, MCAN/FR/87/RM1, MHOM/DZ/82/LIPA59, MHOM/TN/80/IPT1, MHOM/FR/78/LEM75, MHOM/DZ/83/LIPA96, MHOM/FR/88/LEM1265
		L. major	*L. arabica* (5)	MPSA/SA/83/JISH220, MCAN/SA/84/MD84, MPSA/SA/84/JISH238, MPSA/SA/84/JISH231, MPSA/SA/83/JISH224
			L. gerbilli (3)	MRHO/CN/60/GERBILLI, MRHO/SU/87/E11, MRHO/SU/88/KD/88984
			L. major (7)	MHOM/IL/80/FRIEDLIN2, MHOM/IL/2008/LRCL1350, MHOM/MA/81/LEM265, MHOM/MA/2003/LEM4685, MRHO/SU/59/Pstrain, MHOM/TN/2001/LEM4286, MHOM/SU/73/5ASKH
			L. turanica (6)	MMEL/SU/79/MEL, MHOM/SU/64/VL, MRHO/SU/65/VL, MRHO/SU/74/95A, MRHO/SU/95/T9562R, MRHO/SU/91/T/91011L TM
		L. mexicana	*L. amazonensis* (4)	MHOM/BR/72/M1845, MHOM/BR/73/M2269, MHOM/GF/2002/LAV003, MHOM/VE/2004/OHN/15
			L. aristidesi (1)	MORY/PA/69/GML3
			L. garnhami (1)	MHOM/VE/76/JAP78
			L. mexicana (4)	MHOM/BZ/82/BEL21, MNYC/BZ/62/M379, MHOM/VE/2004/CAP04, MHOM/HN/2002/LLM/1162
			L. pifanoi (1)	MHOM/VE/57/LL1
		L. tropica	*L. aethiopica* (6)	MHOM/ET/72/L100, MHOM/ET/91/KASSAYE, MHOM/ET/2004/LPN241, MHOM/JO/2006/LSL103, MHOM/ET/83/10383, MHOM/ET/89/LEM151
			L. killicki (4)	MHOM/DZ/2011/LIPA283, MHOM/TN/80/LEM163, MHOM/TN/80/LEM180, MHOM/TN/86/LEM904/CL
			L. tropica (5)	MHOM/SU/74/K27, MHOM/GR/80/GRL35, MHOM/MA/95/LEM3015, MHOM/MA/2008/W38, MRAT/IQ/72/ADHANIS1
	Viannia	*L. braziliensis*	*L. braziliensis* (4)	MHOM/BR/75/M2904, MHOM/BR/82/LTB12JULY82, MHOM/GF/2003/LAV008, MHOM/BR/75/M2903
			L. peruviana (5)	MHOM/00/2007/TIM24, MHOM/PE/84/LC26, MHOM/PE/84/UN56, MHOM/PE/84/L13A, MHOM/PE/84/L9
		L. guyanensis	*L. guyanensis* (5)	MHOM/GF/98/LEM3657, MHOM/GF/2003/LEM4570, MHOM/GF/2004/LAV016, MHOM/GF/2005/LPC410, MHOM/GF/79/LEM85
			L. panamensis (6)	MHOM/CR/2004/TIM13, MHOM/CR/2007/TIM23, MHOM/00/2004/LEI27, MHOM/EC/90/TERESA, MHOM/CO/89/UA403, MHOM/CR/2001/LCB29A
			L. shawi (1)	MCEB/BR/84/M8408
		L. lainsoni	*L. lainsoni* (6)	IUBI/BR/2000/M12025, MCUN/BR/85/M9342, MHOM/BR/81/M6426, MHOM/PE/2013/LEM6459, MHOM/EC/93/489, MHOM/GF/2002/LEM4449
			L. naiffi (7)	MDAS/BR/79/M5533, MHOM/GF/LCB41, MHOM/GF/2006/LEM5108, MHOM/GF/2005/LEM5109, MHOM/GF/95/LBC1096, MHOM/00/94/CRE58, MHOM/00/99/TIM1
	Sauroleishmania	*Sauroleishmania*	*L. adleri* (1)	RLAT/KE/54/HU1433
			L. gymnodactyli (1)	RGYM/SY/64/LV247
			L. hoogstraali (1)	RLIZ/SD/2000/LV31
			L. tarentolae (3)	RTAR/DZ/39/TARVI, RTAR/FR/77/LEM112, RTAR/FR/78/LEM126
Paraleishmania		*L. herreri*	*L. colombiensis* (2)	IGOM/PA/85/E58234, IHAR/CA/86/CL500
			L. deanei (2)	MCOE/BR/74/M2674, MCOE/BR/75/M2808
			L. equatoriensis (2)	MCHO/EC/82/LSP1.2, MCHO/EC/82/LSP1
			L. hertigi (1)	MCOE/PA/65/C8

[a]The total number of isolates was 121.

these samples). In each laboratory, the MS spectra of the allotted samples (four replicates for each sample) from the panel were used for sample identification with the MSI application and the reference MSL. For each submitted spectrum, the application gave a similarity score that includes three subscores (identification to the species level [score A], identification to the complex level [score B], and identification in a different complex [score C]). Each subscore ranged from 0 to 100 (100 indicates a perfect match

TABLE 2 List of *Leishmania* species tested by the 4 centers and the gold-standard method used[a]

Panel description	No. of isolates tested (method)				
	Montpellier	Cayenne	Barcelona	Marseille	Total
L. adleri	1 (MLEE)				1
L. aethiopica	2 (MLEE)				2
L. amazonensis		1 (RFLP)	1 (*hsp70* and *rpoIILS*)		2
L. archibaldi	1 (MLEE)				1
L. braziliensis	6 (MLEE), 2 (*rpoIILS*)	4 (*rpoIILS*)	7 (*hsp70* and *rpoIILS*)	1 (MLEE)	20
L. deanei	1 (MLEE)				1
L. donovani	3 (MLEE)		4 (*hsp70* and *rpoIILS*)		7
L. enriettii	1 (MLEE)				1
L. gerbilli	1 (MLEE)				1
L. guyanensis	3 (MLEE), 51 (*rpoIILS*)	15 (*rpoIILS*), 1 (RFLP)	1 (*hsp70* and *rpoIILS*)	1 (MLEE)	72
L. gymnodactyli	1 (*rpoIILS*)				1
L. infantum	1 (MLEE)		26 (*hsp70* and *rpoIILS*)	47 (MLEE)	74
L. killicki				3 (MLEE)	3
L. lainsoni	3 (MLEE), 1 (*rpoIILS*)				4
L. major	3 (MLEE), 3 (*rpoIILS*)		4 (*hsp70* and *rpoIILS*)	7 (MLEE)	17
L. mexicana	4 (MLEE)		1 (*hsp70* and *rpoIILS*)		5
L. naiffi	1 (*rpoIIILS*)	1 (RFLP)			2
L. panamensis			1 (*hsp70* and *rpoIILS*)	1 (MLEE)	2
L. peruviana	4 (MLEE)			1 (MLEE)	5
L. pifanoi	1 (MLEE)			1 (MLEE)	2
L. tropica	2 (MLEE), 1 (*rpoIILS*)		2 (*hsp70* and *rpoIILS*)	1 (MLEE)	6
L. turanica	1 (MLEE)			1 (MLEE)	2
Trypanosoma brucei	2				2
Herpetomonas	2				2
Crithidia	2				2
Endotrypanum	1				1
Bacteria	10 (16S rRNA)				10
Fungi	20 (ITS2)				20
Total	135	22	47	64	268

[a]ITS2, internal transcribed spacer 2.

between the compared spectra) (Fig. 1). For the 37 outgroup controls (*Herpetomonas*, *Crithidia*, *Endotrypanum*, bacteria, and fungi), no match with *Leishmania* was obtained, and the similarity score was systematically <17 (data not shown). The MSI application results were then compared with those obtained with the reference identification method used in that laboratory (Table 2). Overall, there was good concordance between methods; however, weakness in species differentiation, particularly within the *L. braziliensis*, *L. guyanensis*, and *L. donovani* complexes, was observed (Table 3). Specifically, only 6 (32%) of the 20 *L. braziliensis* isolates were well classified. However, among the 31 misidentified samples, only 1 was a wrong identification at the complex level and corresponded to 1 *L. braziliensis* isolate that was identified as *L. guyanensis* (Table 3).

Score threshold assessment. Analysis of the three similarity subscores for each sample (Fig. 1) relative to the results obtained with the reference methods indicated that most scores corresponding to a correct identification to the species or complex level were >35. However, the distinction between correct species identification and correct complex identification was more difficult to determine, because there was a clear overlap of the two score distributions (Fig. 1). Therefore, 15 different similarity score thresholds were tested, and positive predictive values (PPVs) (i.e., the proportion of true-positive results) were calculated for each threshold by taking into account only the highest similarity score among the four replicates for each sample (Table 4). The PPV for complex identification was always above 0.99, regardless of the considered threshold. Conversely, it was more difficult to distinguish between species belonging to the same complex, as shown by the PPV results at the species level. The number of misidentifications, particularly at the species level, decreased with higher threshold scores, whereas the number of unidentified strains (with similarity scores below the threshold) increased (Table 4). For instance, with a threshold of 40, all samples were

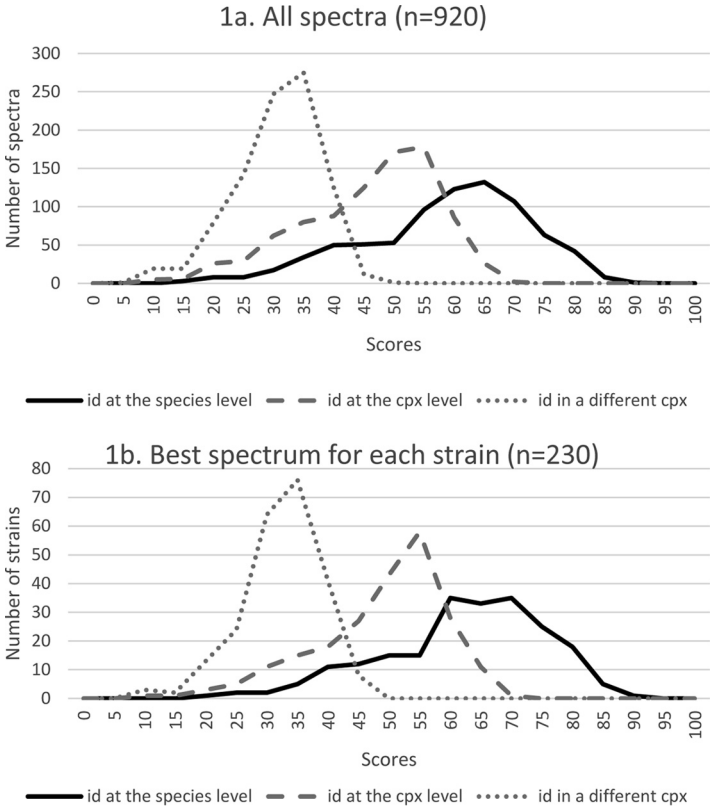

FIG 1 Distribution of the three similarity subscores for all spectra (four replicates for each isolate) (a) and for the spectrum with the best score for each isolate (b). id, identification; cpx, complex. The validation panel included 231 *Leishmania* isolates, but only 230 were identified by the MSI application.

correctly identified to the complex level (PPV = 1), but 13 isolates remained unidentified. These samples corresponded to five correct species identifications, seven correct complex identifications, and one wrong identification.

Culture conditions and testing of parasite concentrations. To identify the best conditions for MALDI-TOF analysis, three strains from the CRB-Leish collection were used for comparing different culture media, parasite concentrations, and culture durations. Species identification for all three strains was obtained with all tested culture media (RPMI, SDM-79, and Schneider media). The best identification scores were obtained when MALDI-TOF analysis was performed with cultures of 3×10^6 *Leishmania*

TABLE 3 List of the 31 misidentifications by MALDI-TOF MS and MS scores obtained

Organism identified by reference method (no. of isolates)	Organism identified by MALDI-TOF MS	MS score(s)
L. braziliensis (13)	*L. peruviana*	32.45, 35.17, 36.45, 38.42, 38.71, 43.49, 47.94, 48.44, 54.44, 56.24, 59.96, 65.8, 66.63
L. braziliensis (1)	*L. guyanensis*	36.48
L. peruviana (1)	*L. braziliensis*	63.83
L. guyanensis (7)	*L. panamensis*	41.08, 41.66, 41.95, 42.78, 43.14, 51.73, 60.21
L. panamensis (1)	*L. guyanensis*	55.8
L. mexicana (1)	*L. pifanoi*	69.57
L. pifanoi (1)	*L. mexicana*	55.79
L. deanei (1)	*L. hertigi*	43.84
L. donovani (2)	*L. archibaldi*	45.04, 53.57
L. infantum (3)	*L. archibaldi*	40.49, 47.22, 52.78

TABLE 4 Identification capacities with the various tested score thresholds

Score threshold	No. of isolates identified correctly to the taxon level	No. of isolates identified correctly to the complex or species level	No. of isolates with incorrect identification	No. of isolates for which the score threshold was not reached	PPV	
					Taxon level	Complex level
20	200	0	1	0	0.86	0.99
25	198	30	1	2	0.86	0.99
30	198	30	1	2	0.86	0.99
35	197	29	1	4	0.86	0.99
40	193	25	0	13	0.89	1
45	184	17	0	30	0.91	1
50	171	13	0	47	0.92	1
55	159	9	0	63	0.94	1
60	138	5	0	88	0.96	1
65	100	3	0	128	0.97	1
70	63	0	0	168	1	1
75	34	0	0	197	1	1
80	13	0	0	218	1	1
85	2	0	0	229	1	1
90	0	0	0	231		

parasites/ml in the exponential phase of culture growth, regardless of the time required to obtain this concentration.

MS data acquisition. In terms of identification performance, no difference was observed when mass spectra were acquired by using the Microflex or Autoflex instrument (Bruker Daltonics).

Online accessibility. The four participating centers tested the online accessibility of the MSI application. The delay between the query submission and the Excel report acquisition for inputs containing up to 96 spectra was $<$1 min for all centers. Performances were equivalent with the Mac and Windows operating systems using any of the most used Web browsers (Google Chrome, Mozilla Firefox, Safari, and Internet Explorer).

DISCUSSION

The purpose of this study was to create and test the reliability of a database for *Leishmania* identification by MALDI-TOF MS using a free Web-based application to meet researchers' and public health needs. The database included spectra from 10 *Leishmania* complexes representing 33 species, thus covering the main diversity of this genus. Access to the MSI application requires an account and acceptance of a policy agreement. The account is password protected, and access codes are delivered for free after a discussion of the nature of the project, with the aim of ensuring that users are using the application for public health or scientific purposes.

Our results show that neither the medium used nor the time spent to reach the appropriate promastigote concentration significantly influenced the quality of spectra and, thus, the identification. The important point is to obtain a promastigote concentration of 3×10^6 parasites/ml from an exponential-phase culture. Once the sample is prepared, at least four replicates per strain are required, as previously recommended by Cassagne et al. (14). As already observed in many studies, the interpretation of results depends on the chosen score threshold (15, 16). In this study, the lower threshold considered was 20 because our experience with other organisms, such as fungi, indicates that nonspecific identifications are frequent below this threshold (17). With this threshold, 230 of the 231 *Leishmania* strains were correctly identified at least to the complex level. By increasing the threshold up to 40, the frequency of misidentification at the species level was greatly reduced; however, the number of samples that did not reach the threshold increased, despite these samples being correctly identified in most cases. In real life, the threshold choice can be influenced by many factors, and each user will have to weigh the advantages and disadvantages of each threshold, depending on the required level of accuracy of identification. Nevertheless, the MSI application allows

obtaining reliable *Leishmania* identifications by MALDI-TOF MS in most cases. Moreover, as already seen with other databases, its reliability should increase progressively with the addition of new references (18).

Considering the complexes frequently involved in CL, we observed that *L. major* and *L. tropica* identifications were 100% correct. Moreover, our identification system performed well in differentiating *L. killicki* from *L. tropica* strains, although it was recently proposed that they could be considered synonymous (19). Conversely, many more incorrect species identifications were obtained for the *L. braziliensis* complex, which is composed of *L. braziliensis* and *L. peruviana*. According to the literature and our experience, very few differences are observed between these species using MLEE (20) or molecular methods (20–23). As the epidemiological data, clinical presentations, and treatments of CL caused by these two species are quite similar, identification to the complex level remains adequate. When considering a threshold value below 40, one *L. braziliensis* isolate was misidentified by the MSI application as *L. guyanensis*, a species belonging to the same subgenus but to a different complex. It is known that the *L. braziliensis* complex is very polymorphic, with a high recombination level, and is closely related to the *L. guyanensis* complex (24). Only one spectrum (although it obtained the highest similarity score) pointed to an identification of *L. guyanensis* (score of 36.48), while the other three replicates identified *L. braziliensis* (scores of 31.22 and 31.46) and *L. panamensis* (score of 31.98). Thus, in some cases, a general view of the results of the four scores could provide a better approach. For the *L. guyanensis* complex, which includes the species *L. guyanensis*, *L. panamensis*, and *L. shawi*, 100% and 90.4% of the identifications were correct at the complex and species levels, respectively. As for *L. braziliensis* and *L. peruviana*, *L. shawi* and *L. panamensis* are considered to be two clusters inside the *L. guyanensis* complex rather than two different species (21, 24–26).

As CL is a heterogeneous entity, and no therapeutic option is currently effective for all clinical forms, the species involved and the geographic area of contamination guide treatment decisions. Indeed, metastatic extension to the mucosa occurs in 1 to 3% of patients for some species. Fortunately, complex identification is sufficient to guide treatment management (5, 27, 28). Therefore, MALDI-TOF MS is adequate for the first-line identification of strains involved in CL or mucocutaneous leishmaniasis.

Some misidentifications also occurred within the *L. donovani* complex, which currently includes three species: *L. donovani*, *L. infantum*, and *L. archibaldi*. In our study, it was possible to identify 96% of *L. infantum*, 71% of *L. donovani*, and all *L. archibaldi* strains to the species level. All misidentified *L. donovani* isolates were erroneously identified as *L. archibaldi*. According to MLEE, the species *L. archibaldi* is characterized by a single enzyme, and some molecular studies suggest that the *L. donovani* complex is more likely to be a continuum in which *L. archibaldi* stands between the two other species (29). Therefore, some authors attribute the category of subspecies to *L. infantum* and *L. donovani* (30), and it is widely accepted that *L. archibaldi* should not be considered a real species (31–34). Anyway, there is no impact on therapeutic management because this complex is responsible for visceral forms of leishmaniasis, for which treatment does not depend on the species but on the therapeutic agents available in that country.

Although work is in progress to improve the sensitivity of the MSI application for the *L. braziliensis*, *L. guyanensis*, and *L. donovani* complexes, the clinical context and epidemiological data also need to be considered to confirm *Leishmania* identification. Therefore, the medical biologist should remain completely responsible for the diagnostic procedure.

Overall, the MSI application proved to be a solid identification tool, leading to identification results comparable to those obtained with reference techniques but faster. As shown by the large experience acquired with other microorganisms, the addition of new references will improve current performances; therefore, this identification tool will be further developed with the user community's help. The database could be regularly enriched and updated by users, by adding spectra representing new or rare species. Sharing access to a database, as we propose, should facilitate the

implementation of studies requiring the identification of various organisms, by making available a common identification tool. Moreover, it provides an opportunity to implement collaborative research between groups working on related topics. Beyond the specific problem of identifying *Leishmania*, the approach presented here has also been applied to other organisms such as fungi (35), and we hope that similar initiatives will be implemented to build other identification systems, based on the collective construction of databases by scientific teams.

MATERIALS AND METHODS

Sample preparation. (i) *Leishmania* cultures. *Leishmania* cultures were grown in RPMI (Sigma) and Schneider medium (Sigma) supplemented with 10% heat-inactivated fetal calf serum or in SDM-79 medium at 25°C for 4 to 7 days.

(ii) Sample preparation for MALDI-TOF analysis. Five milliliters of a promastigote culture containing about 3×10^6 parasites/ml in the exponential phase was harvested by centrifugation (10 min at 3,000 $\times$ *g*) and washed three times with 10 ml of 0.9% NaCl. Promastigote pellets were resuspended in one drop (approximately 20 μl) of 0.9% NaCl. One microliter of the solution was deposited onto the steal target and, once dried, covered with 1 μl of an α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Sigma-Aldrich, Lyon, France). Ten replicates were prepared for every isolate included in the MSL, and four replicates were performed for each isolate in the panel to be tested.

(iii) Mass spectrum acquisition. Mass spectra were acquired with a Microflex LT instrument (Bruker Daltonics) (three centers, Marseille, Montpellier, and Cayenne, France) or an Autoflex II TOF-TOF instrument (Bruker Daltonics) (one center, Barcelona, Spain) with the default acquisition parameters recommended by Bruker, as described previously by Cassagne et al. (11).

Identification system. (i) Reference database. A total of 121 *Leishmania* isolates (Table 1) to be included in the MSL were selected from the CRB-Leish collection. All strains were previously identified by using MLEE as described previously by Rioux et al. (36), and for 50 isolates, species identification was also confirmed by multilocus sequence typing (29). Raw mass spectra were obtained with the Microflex LT instrument (Bruker Daltonics) in Montpellier and used to construct the database. The assignment of each isolate to a group/complex/species was based on the taxonomic classification proposed recently by Akhoundi et al. (2).

(ii) Identification system. The MSL was implemented in a Web-based application designed for microbial mass spectrum identification (MSI application).

For identification, the raw mass spectra of a sample were compressed into a .zip file and submitted to the Web application. As soon as they were uploaded, raw spectra were subjected to several mathematical operations to eliminate background noise, smooth intensity values, and identify relevant peaks. A series of peak values was automatically built and compared with the peak values of all the reference spectra included in the database. The similarity between the spectrum to be identified and each reference spectrum was rated on a scale ranging from 0 to 100 (100 indicates a perfect match between the compared spectra). To establish the similarity level, the identification system relies on original algorithms, independently from the FlexAnalysis and MaldiBioTyper software provided by Bruker Daltonics. Many samples can be analyzed in one request provided that the number of spectra remains below a few hundred. Results can be exported as Excel files with a similarity score value for each spectrum. Reports may be stored in the user's account.

Implementation of the identification system. (i) Tested panel. The panel of isolates used to test the online MSI application and the MSL database included 268 strains: 231 *Leishmania* isolates and 37 outgroup controls (Table 2). The panel was constituted by strains from the four laboratories that participated in the study (four subpanels): (i) 47 isolates were obtained in Barcelona (Secció de Parasitologia, Facultat de Farmàcia i Ciències de l'Alimentació, Universitat de Barcelona) and analyzed at the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain); (ii) 22 isolates were obtained prospectively in French Guyana (Laboratoire de Parasitologie-Mycologie, Centre Hospitalier Universitaire de Cayenne); (iii) 64 strains, 31 originating from Maghreb countries (12 from the Institut Pasteur of Tunis, 19 from the Institut Pasteur of Alger) and 33 isolated in Marseille, were analyzed in Marseille, France; and (iv) 98 strains were selected from CRB-Leish in Montpellier, France, to increase the diversity of species and countries of origin of the strains. For each subpanel, various reference identification methods were used as gold standards, depending on the technique available in the laboratory, including MLEE (36), RFLP, sequencing of the heat shock protein 70 gene (*hsp70*) (22), or sequencing of the *rpoIILS* gene (2).

(ii) MSI performance and score threshold assessments. Isolates from the panel were identified by using the MSI application and the MSL. The analysis report for each spectrum indicates (i) the species with the highest similarity score (this can be considered the isolate identification result) (score A); (ii) the best score obtained for a different species of the same complex, according to the taxonomy reported by Akhoundi et al. (2) (score B); and (iii) the best score obtained for a species in a different complex (score C). The identification results were compared to the results obtained with the gold-standard/reference method for each isolate. The threshold was established in two steps. First, the three scores for each spectrum, when available, were plotted to visualize their overlap (Fig. 1). Next, the performance of the identification system was tested with different thresholds, from 20 to 90, with increments of 5 units for each cutoff (Table 4). Threshold values below 20 were not considered because our previous experience with fungi (17) showed that misidentifications might occur at values below this threshold.

As suggested previously by Normand et al. (17), four replicates for each isolate were deposited, but only the replicate with the highest score was selected, and the identification corresponds to the one obtained for this replicate.

(iii) Testing of culture conditions (medium and duration) and parasite concentrations. To identify the best culture conditions and parasite concentration for MS identification, three strains from CRB-Leish were selected: *L. donovani* MHOM/IN/2003/LEM4537, *L. major* MHOM/TN/93/LPN89, and *L. guyanensis* MHOM/GF/2004/LBC45. Three different media were tested: SDM-79, RPMI, and Schneider medium supplemented with 10% heat-inactivated fetal calf serum. Isolates were cultured at 25°C. Testing (in triplicate) was done on days 4, 6, 8, and 10 of culture by using five concentrations (from 10^2 to 10^6 parasites/ml).

(iv) Online accessibility. The four centers tested MSI application accessibility and capacities for Mac and PC systems (Windows 7 and Windows 10) with several Web browsers (Google Chrome, Mozilla Firefox, Safari, and Internet Explorer).

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