Am. J. Trop. Med. Hyg., 66(2), 2002, pp. 152–156

RANDOM AMPLIFIED POLYMORPHIC DNA TECHNIQUE FOR IDENTIFICATION AND DIFFERENTIATION OF OLD WORLD *LEISHMANIA* SPECIES

IKRAM GUIZANI, KOUSSAY DELLAGI, AND RIADH BEN ISMAÏL

Laboratoire d'Epidémiologie et d'Ecologie Parasitaire, Institut Pasteur de Tunis, Tunisia; Laboratoire d'Immunologie, Institut Pasteur de Tunis, Tunisia

Abstract. The random amplified polymorphic DNA technique may be used to explore parasite DNA polymorphisms. We assessed its applicability to identification of Old World *Leishmania* species. A set of 6 random decamer primers (A1, A4, A5, A7, A10, and A15) was applied to a panel of DNA from 57 representatives of different Old World *Leishmania* species. The amplification profiles allowed discrimination among species belonging to different taxonomic complexes. Two criteria were used to analyze the profiles: the presence of consistent amplicons at the same electrophoretic position for isolates of the same species, and the presence of distinct amplicons for isolates of different species. Three primers—A1, A7 and A10—rendered such products.

INTRODUCTION

Parasites belonging to the genus *Leishmania* have wide geographic distribution and cause different clinical manifestations, including cutaneous, mucocutaneous, and visceral pathology. Different *Leishmania* species may be encountered within the same geographic area. Precise identification of these species is thus important for clinical and epidemiologic reasons. *Leishmania* parasites have similar morphology and sometimes cause similar clinical manifestations; therefore, differentiation among species requires molecular techniques such as isoenzyme electrophoresis, DNA probe hybridization, and polymerase chain reaction (PCR)-based techniques.[1–5]

The random amplified polymorphic DNA (RAPD) technique uses random oligomers to amplify genomic DNA and thus does not necessitate any prior knowledge on organism's genomic sequences.[6] It has been applied to genetic studies of mice,[7] molecular ecology,[8] and identification of differentially expressed sequences.[9–10] It has also been used to study polymorphisms of microorganisms of medical importance such as trypanosomes,[11–13] *Plasmodium* species,[14,15] and *Toxoplasma gondii.*[16] In the case of *Leishmania,* RAPD studies mostly aimed to assess the technique to establish and estimate the genetic relationship among sets of taxa, species, and isolates.[17–19] The present study sought to assess the suitability of RAPD for *Leishmania* parasite species identification by examination of the existence of consistent and diagnostic electrophoretic profiles among Old World *Leishmania* species.

MATERIALS AND METHODS

Parasites and DNA. Fifty-seven *Leishmania* isolates were obtained from reference centers in Montpellier, France (J. A. Rioux, F. Pratlong), London, UK (D. A. Evans), and Rome, Italy (L. Gradoni) and from the Royal Tropical Institute, Amsterdam, the Netherlands (G. J. J. M. van Eys). Of these, 43 were typed by isoenzyme analysis by the donors. Thirty isolates were selected from field collections in Tunisia. The panel included representatives of the species *Leishmania arabica, Leishmania aethiopica, Leishmania donovani, Leishmania infantum, Leishmania killicki, Leishmania major, Leishmania tropica, Leishmania turanica,* and *Sauroleishmania tarentolae.* The denomination of species or complexes used in this work is based on the classification of Rioux and others.[1]

The parasites were isolated from different hosts or vectors in 14 different countries in the Old World and corresponded to at least 21 zymodemes. The panel also included one isolate of *Leishmania chagasi* from the New World (Table 1). The organisms were cultured in RPMI 1640 supplemented with 15% fetal calf serum. Genomic DNA was obtained as described previously.[3] Parasite species identification was reconfirmed by use of nuclear probes pDK20 and pDK10, which exhibit species-specific hybridization patterns.[2,3]

Amplification via RAPD. A set of 6 decamers was used: A1 (CAGGCCCTTC), A4 (AATCGGGCTG), A5 (AGGGGTCTTG), A7 (GAAACGGGTG), A10 (GTGATCGCAG), and A15 (TTCCGAACCC) (Operon, Alameda, CA). The amplification protocol was described previously.[13] The reaction mix included 0.1 mM of each dNTP, 0.3–0.4 μM primer, 1.5 U Taq DNA polymerase (Boehringer, Manheimm, Germany), 1× buffer supplied with the enzyme (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), and 20 ng genomic DNA. Amplification was processed for 45 cycles that comprised successively a 1-min denaturation step at 94°C, a 1-min annealing step at 36°C, and a 2-min elongation at 72°C. Final elongation was performed for 7 min at 72°C. Polymerase chain reaction products were separated by 1.6% agarose gel electrophoresis and visualized by ultraviolet light after staining with ethidium bromide. Interpretation of the patterns was based on the size and on the presence or absence of amplified DNA bands.

RESULTS

The RAPD products. One to 10 RAPD products were observed with different profiles. According to taxa, some were simple, comprising 1–2 intense bands, whereas other profiles were more complex. For the latter, the amplified DNAs included strong and weak intensity and fuzzy bands. Amplicon size ranged 3–0.2 kb. Reproducibility was assessed by means of different DNA preparations or by means of the same DNA preparations of some isolates. The profile of amplicons was reproducible in most cases. Primer A5 was most prone to variations.

Discrimination of parasites and species identification. The profiles obtained with each primer were compared with

TABLE 1
Selection of *Leishmania* parasite isolates*

Number	WHO code	Zymodeme	Species
1	MHOM/TN/80/IPT1	MON-1	*L. infantum*
2	MHOM/TN/87/KA 412	MON-1	*L. infantum*
3	MHOM/TN/87/KA 413	MON-1	*L. infantum*
4	MHOM/TN/87/KA 390	MON-1	*L. infantum*
5	MHOM/TN/88/KA 439	MON-1	*L. infantum*
6	MHOM/TN/88/KA 455	MON-1	*L. infantum*
7	MHOM/TN/88/Aymen	MON-1	*L. infantum*
8	MHOM/TN/89/Afef	MON-1	*L. infantum*
9	MHOM/TN/88/Nabil	MON-1	*L. infantum*
10	MHOM/TN/89/Moalla	–	*L. infantum*
11	MHOM/TN/90/OS	–	*L. infantum*
12	MHOM/TN/89/KA524	–	*L. infantum*
13	MHOM/TN/92/LV08	–	*L. infantum*
14	MHOM/TN/92/LV16	–	*L. infantum*
15	MHOM/TN/93/OT	–	*L. infantum*
16	MCAN/TN/87/CN 5-SBZ	MON-1	*L. infantum*
17	MCAN/TN/87/CN 26-SBZ	MON-1	*L. infantum*
18	MCAN/TN/87/CM 30-SBZ	MON-1	*L. infantum*
19	MCAN/TN/89/CN 33	–	*L. infantum*
20	MCAN/TN/87/CN 49-SBZ	MON-1	*L. infantum*
21	MCAN/TN/87/CN 52-SBZ	MON-1	*L. infantum*
22	MCAN/TN/87/CN 64-SBZ	MON-1	*L. infantum*
23	MCAN/TN/87/CN 65-SBZ	MON-1	*L. infantum*
24	MCAN/TN/88/CN 145-SBZ	MON-1	*L. infantum*
25	MCAN/TN/89/Alm220	–	*L. infantum*
26	MCAN/GR/82/LEM 497	MON-1	*L. infantum*
27	MCAN/TN/92/92BMCN01	–	*L. infantum*
28	MCAN/TN/92/92BMCN02	–	*L. infantum*
29	MCAN/TN/92/92BMCN04	–	*L. infantum*
30	MHOM/DZ/87/Lem 1227	MON-1	*L. infantum*
31	MHOM/DZ/83/LIPA120	MON-34	*L. infantum*
32	MHOM/ES/81/Palma	MON-1	*L. infantum*
33	MHOM/ES/87/Lombardi	LON-49	*L. infantum*
34	MCAN/IT/84/ISS29	MON-1	*L. infantum*
35	MHOM/FR/62/LRC-L47	–	*L. infantum*
36	MHOM/BR/74/PP 75	MON-1	*L. chagasi*
37	MHOM/IN/80/DD8	MON-2	*L. donovani*
38	MHOM/SD/68/1S	–	*L. donovani*
39	MHOM/SU/73/5-ASKH	MON-4 LON-1	*L. major*
40	MRHO/SU/59/P-strain	MON-4 LON-1	*L. major*
41	MPSA/SA/86/PSM-3125	MON-26 LON-4	*L. major*
42	MHOM/SA/84/KFUH-7532	LON-65	*L. major*
43	MHOM/IL/67/Jericho II	MON-26 LON-70	*L. major*
44	MHOM/SN/00/DK1	MON-26 LON-1	*L. major*
45	MHOM/IL/83/IL 32	MON-68 LON-1	*L. major*
46	MPSA/TN/87/Ron 44	MON-25	*L. major*
47	MMER/IN/73/GTBM	MON-23 LON-5	*L. major*
48	MRHO/SU/74/95-A	MON-64	*L. turanica*
49	MPSA/SA/84/Jisha 238	LON-64	*L. arabica*
50	MHOM/ET/72/L100	MON-14 LON-27	*L. aethiopica*
51	MHOM/TN/80/LEM 904	MON-8	*L. killicki*
52	MHOM/SU/74/SAF-K27	MON-60 LON-12	*L. tropica*
53	MRAT/IQ/73/Adhanis I	MON-5 LON-15	*L. tropica*
54	MCAN/IN/71/DBKM	MON-62 LON-21	*L. tropica*
55	MHOM/IQ/73/Bumm30	LON-17	*L. tropica*
56	MHOM/IQ/76/BAG 9	MON-53 LON-23	*L. tropica*
57	IMIN/IT/86/MIN1	–	*S. tarentolae*

* Host and geographical origin (WHO code) are summarized together with zymodeme attribution whenever available. MON and LON correspond to zymodemes attributed by the reference centers in Montpellier and London, respectively. Parasite identification was confirmed for all *Leishmania* isolates using nuclear DNA probes. Country abbreviations are shown as specified by WHO recommendations. SU = ex–Soviet Union; WHO = World Health Organization.

respect to taxonomic classification of the isolates within a species or species complex. Two criteria were taken into account: the consistent presence of amplified DNA bands at the same electrophoretic position for isolates of a same species; and the discrimination between isolates belonging to different species. This allowed analysis of the results obtained with the A1, A7, and A10 primers. These profiles are illustrated in Figure 1.

Table 2 summarizes the electrophoretic position of the diagnostic bands identified for each primer and species tested. A major DNA product was observed for most species with the primer A1 (Figure 1C). The sizes were consistently es-

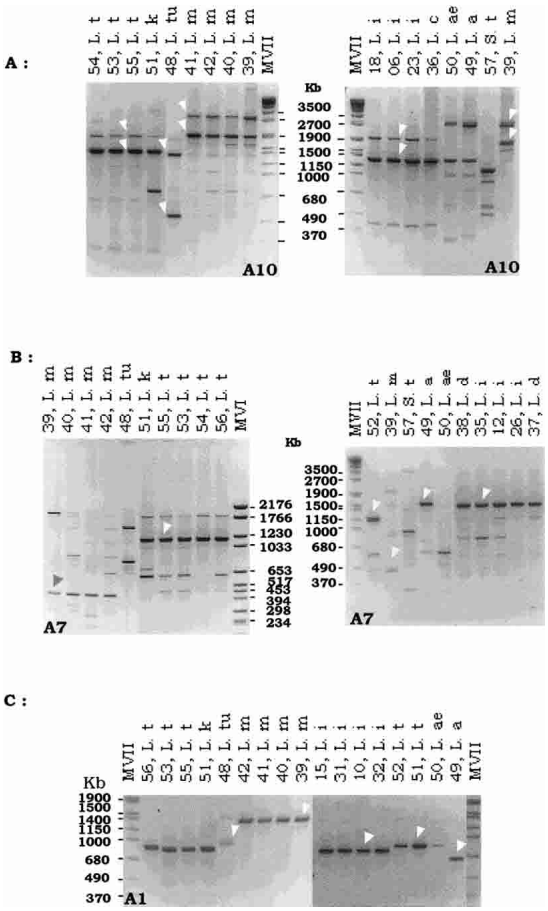

FIGURE 1. Random amplified polymorphic DNA (RAPD) amplification profiles observed with A10 **(A)**, A7 **(B)**, and A1 **(C)** primers. The figure illustrates different levels of sharpness and intensities. On the basis of electrophoretic position, products were consistently observed that are characteristic of parasite species **(arrowheads)**. Each lane corresponds to a DNA isolate, which is identified by the number reported in Table 1 and its species attribution. a = *arabica*; ae = *aethiopica*; c = *chagasi*; d = *donovani*; i = *infantum*; k = *killicki*; L. = *Leishmania*; m = *major*; t = *tropica*; tu = *turanica*; S. t = *S. tarentolae.* MVI and MVII = DNA size markers VI and VII (Boehringer, Mannheim, Germany).

timated to be 1.2, 0.8, and 0.9 kb for all isolates of *L. major, L. infantum,* and *L. tropica,* respectively. Furthermore, *L. arabica* and *L. turanica* were shown to produce fragments of 0.65 and 0.95 kb, respectively. *Leishmania aethiopica* shared the 0.9-kb fragment with *L. tropica.* Consistent amplification of one intense band was also observed with the A7 primer (Figure 1B). A band of 1.4, 0.45, and 1.2 kb allowed discrimination among *L. infantum, L. major,* and *L. tropica,* respectively. *Leishmania arabica* and *L. aethiopica* reference isolates amplified a product at 1.5 and 0.55 kb, respectively. It is interesting to note that *L. turanica* DNA yielded 2 major products of 1.6 and 0.79 kb, which allowed its differentiation from *L. major* DNA. Within *L. major,* one exception was observed with one isolate (Ron44), which did not amplify the 0.45-kb product. In the case of A10 primer, 2 bands were consistently observed for each species. Their presence discriminated among the taxa (Figure 1A). The 1.9- and 1.3-kb DNA bands were diagnostic for *L. infantum*; the combinations of 2.7–1.9 kb and 1.6–1.3 kb were diagnostic for *L. major* and *L. tropica,* respectively. An additional 0.83-kb band allowed identification of *L. killicki* among the *L. tropica* complex. The presence of 1.3- and 0.55-kb DNA bands discriminated the *L. turanica* isolate from *L. major.* Both *L. arabica* and *L. aethiopica* DNAs amplified bands at 2.7 and 1.3 kb.

None of the 3 primers allowed distinction among the parasites of the species complex *L. donovani* (*L. infantum, L. chagasi,* and *L. donovani*). With the primers A1 and A7, *L. killicki* shared the same profiles as other parasites of the *L. tropica* complex. With all primers, the *L. tarentolae* isolate presented distinct profiles. Furthermore, pairwise association of RAPD results allowed distinctive identification of parasites of the *L. donovani* complex, *L. tropica* complex, *L. major, L. aethiopica, L. turanica,* and *L. arabica* (Table 2).

DISCUSSION

Unlike other PCR-based assays commonly used for molecular diagnosis, RAPD does not require previous knowledge of nucleotide sequences. In RAPD analysis, only one randomly designed decamer with 60–70% GC content and no self-complementary ends is used to prime amplification. The length of the primer and the relaxed specificity of the reaction conditions are major reasons for the extensive ap-

TABLE 2
Summary of results obtained with the A1, A7, and A10 primers

Species	*n*	Size estimates (in kb) of RAPD products with A1, A7, and A10 primers		
		A1	A7	A10
L. aethiopica	1	0.9	0.55	2.7 and 1.3
L. major	9	1.2	0.45	2.7 and 1.9
L. turanica	1	0.95	1.6 and 0.79	1.3 and 0.55
L. arabica	1	0.65	1.5	2.7 and 1.3
Leishmania donovani complex				
L. donovani	2	0.8	1.4	1.9 and 1.3
L. infantum	35	0.8	1.4	1.9 and 1.3
L. chagasi	1	0.8	1.4	1.9 and 1.3
Leishmania tropica complex				
L. tropica sensu stricto	5	0.9	1.2	1.6 and 1.3
L. killicki	1	0.9	1.2	1.6, 1.3, and 0.83

plication of the RAPD technique to a variety of organisms, including *Leishmania* sp.[6–19] This study aimed to assess RAPD as a simple method for *Leishmania* parasite species identification. The technique requires as little as 20 ng DNA and only a few parasites per reaction. This is less than that required for isoenzyme analysis or DNA probes hybridization. Furthermore, unlike most other PCRs, which necessitate additional steps such as oligonucleotide hybridization, sequencing, or restriction analysis, the RAPD approach directly distinguishes various parasite species from each other. The results are based on direct observation of bands on agarose gels.

Another advantage is that identical reaction conditions can be used with different primers, with different genomic DNA samples, or both. The broad specificity of the primers restricts the use of this technique to isolated parasites. To date, RAPD technique has not been applied to clinical samples but limited to isolated organisms. Isolation and culture of *Leishmania* are sometimes difficult. In some situations, only 50% of the isolates can be propagated in long-term culture. By use of RAPD, promastigotes in early steps of *in vitro* culture could be studied with no need for long-term cultivation. The simple interpretation of results from identification of PCR products in agarose gels is an advantage for laboratories in field settings.

The reaction products obtained by RAPD enabled discrimination among species. Further, the amplification profiles were reproducible, although there were minor differences for bands of faint intensity. The current study benefited from access to 57 parasite isolates representative of all Old World *Leishmania* species,[1] with the exception of *L. archibaldi* and *L. gerbilli.* As illustrated for the A1, A7, and A10 primers, and as illustrated by the reliance on the electrophoretic pattern of the amplicons, concomitant amplification of variable and consistent DNA bands was observed. Our analysis emphasized consistent amplification within a taxon of one or more DNA bands of mobility considered as diagnostic of the species. At this step, with no other knowledge on the nature, sequence, and relatedness of the amplification products, the distinction of the parasites according to the RAPD profiles is carefully considered as merely phenetic.

Two criteria for species diagnosis were considered to be important: the consistent presence of RAPD products of identical electrophoretic position for isolates of the same species, and their ability to discriminate between parasites of different species. The RAPD analysis produced distinctive amplification products among 8 Old World *Leishmania* species tested (*L. aethiopica, L. arabica, L. donovani, L. infantum, L. killicki, L. major, L. tropica,* and *L. turanica*). The A1, A7, and A10 primers allowed differentiation between isolates responsible for cutaneous disease (*L. major, L. tropica, L. aethiopica,* and *L. infantum*) or those causing cutaneous (*L. major, L. tropica,* and *L. aethiopica*) and visceral disease (*L. infantum, L. chagasi,* and *L. donovani*). They could also discriminate between species encountered in the same foci with the same reservoir hosts, such as *L. arabica* and *L. major* or *L. turanica* and *L. major.*[20–21]

Sauroleishmania tarentolae, often sympatric to many *Leishmania* species, was included in this study. Its amplification patterns with the A1, A7, and A10 primers differed from those of *Leishmania.* The discriminative power of each primer between all species assayed was limited in few situations. Indeed, the pairwise association of RAPD results obtained for the A1, A7, and A10 primers was shown to distinctively identify parasites of the *L. donovani* complex, *L. tropica* complex, *L. major, L. aethiopica, L. turanica,* and *L. arabica.* We thus recommend that at least 2 primers be used for species diagnosis. The A10 primer allowed differentiation among parasites of the *L. tropica* complex with an additional intense amplicon for *L. killicki.* The presence of a DNA band at this electrophoretic position should be considered diagnostic for this species after confirmation with additional *L. killicki* isolates. Results observed with only one isolate should be considered preliminary until confirmed with more isolates. Furthermore, the current study did not allow identification of discriminative RAPD products for parasites of the *L. donovani* complex (*L. infantum, L. donovani,* and *L. chagasi*). This may be overcome by increasing the number of primers. A RAPD-based distinction among these parasite species was shown to be possible.[10]

The outcome of a RAPD reaction is highly dependent on parameters that may vary from one laboratory setting to another.[22] This limitation may be overcome by the simplicity of the technique described here. It can be applied to any other arbitrary primers or organisms. It is therefore recommended that these reactions be based on a panel of well-characterized isolates and standardized conditions before installing such studies.

Acknowledgments: We are grateful to A. L. Banuls and M. Tibayrenc for technical advice and for introducing us to the technique, and we are grateful to M. Ben Fadhel, A. Ben Hamouda, A. Zaatour, and R. Hanafi for technical assistance.

Financial support: This investigation received financial support from the Tropical Disease Research Program of the World Health Organization (TDR/ RSG ID 890266) and the Initiative Avicenne of the EC (AVC.CT.920013). I.G. is a recipient of a TDR research training grant (grant 920781).

Authors' addresses: Ikram Guizani and Riadh Ben Ismaïl, Laboratoire d'Epidémiologie et d'Ecologie Parasitaire, Institut Pasteur de Tunis, BP 74, 1002 Tunis Belvédère, Tunisia. Koussay Dellagi, Laboratoire d'Immunologie, Institut Pasteur de Tunis, BP 74, 1002 Tunis Belvédère, Tunisia.

Reprint requests: Ikram Guizani, Laboratoire d'Epidémiologie et d'Ecologie Parasitaire, Institut Pasteur de Tunis, BP 74, 1002 Tunis Belvédère, Tunisia, Telephone: 216-1-792-429, Fax: 216-1-791-833, E-mail: ikram.guizani@pasteur.rns.tn.

REFERENCES

1. Rioux JA, Lanotte G, Serres E, Pratlong F, Bastien P, Perrieres J, 1990. Taxonomy of *Leishmania.* Use of isoenzymes. Suggestions for a new classification. *Ann Parasitol Hum Comp 65:* 111–125.
2. van Eys GJ, Guizani I, Ligthart GS, Dellagi K, 1991. A nuclear DNA probe for the identification of strains within the *Leishmania donovani* complex. *Exp Parasitol 72:* 459–463.
3. Guizani I, Van Eys GJ, Ismaïl RB, Dellagi K, 1994. Use of recombinant DNA probes for species identification of Old World *Leishmania* isolates. *Am J Trop Med Hyg 50:* 632–640.
4. van Eys GJ, Schoone GJ, Kroon NC, Ebeling SB, 1992. Sequence analysis of small subunit ribosomal RNA genes and its use for detection and identification of *Leishmania* parasites. *Mol Biochem Parasitol 51:* 133–142.
5. Degrave W, Fernandes O, Campbell D, Bozza M, Lopes U,

1994. Use of molecular probes and PCR for detection and typing of *Leishmania*—a mini-review. *Mem Inst Oswaldo Cruz 89:* 463–469.
6. Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res 18:* 6531–6535.
7. Welsh J, Petersen C, McClelland M, 1991. Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. *Nucleic Acids Res 19:* 303–306.
8. Hadrys H, Balick M, Schierwater B, 1992. Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol Ecol 1:* 55–63.
9. Murphy NB, Pelle R, 1994. The use of arbitrary primers and the RADES method for the rapid identification of developmentally regulated genes in trypanosomes. *Gene 141:* 53–61.
10. Pogue GP, Lee NS, Koul S, Dwyer DM, Nakhasi HL, 1995. Identification of differentially expressed *Leishmania donovani* genes using arbitrarily primed polymerase chain reactions. *Gene 165:* 31–38
11. Waitumbi JN, Murphy NB, 1993. Inter- and intra-species differentiation of trypanosomes by genomic fingerprinting with arbitrary primers. *Mol Biochem Parasitol 58:* 181–185.
12. Steindel M, Dias Neto E, de Menezes CL, Romanha AJ, Simpson AJ, 1993. Random amplified polymorphic DNA analysis of *Trypanosoma cruzi* strains. *Mol Biochem Parasitol 60:* 71–79.
13. Tibayrenc M, Neubauer K, Barnabe C, Guerrini F, Skarecky D, Ayala FJ, 1993. Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. *Proc Natl Acad Sci U S A 90:* 1335–1339.
14. Carlton JM, Howard J, Jensen JB, Walliker D, 1995. A rapid technique for the detection of DNA polymorphisms in *Plasmodium. Exp Parasitol 80:* 163–166.
15. Howard J, Carlton JM, Walliker D, Jensen JB, 1996. Use of random amplified polymorphic DNA (RAPD) technique in inheritance studies of *Plasmodium falciparum. J Parasitol 82:* 941–946.
16. Guo ZG, Johnson AM, 1996. DNA polymorphisms associated with murine virulence of *Toxoplasma gondii* identified by RAPD-PCR. *Curr Top Microbiol Immunol 219:* 17–26.
17. Noyes HA, Belli AA, Maingon R, 1996. Appraisal of various random amplified polymorphic DNA–polymerase chain reaction primers for *Leishmania* identification. *Am J Trop Med Hyg 55:* 98–105.
18. Schonian G, Schweynoch C, Zlateva K, Oskam L, Kroon N, Graser Y, Presber W, 1996. Identification and determination of the relationships of species and strains within the genus *Leishmania* using single primers in the polymerase chain reaction. *Mol Biochem Parasitol 77:* 19–29.
19. Motazedian H, Noyes H, Maingon R, 1996. *Leishmania* and *Sauroleishmania*: the use of random amplified polymorphic DNA for the identification of parasites from vertebrates and invertebrates. *Exp Parasitol 83:* 150–154.
20. Peters W, Elbihari S, Evans DA, 1986. *Leishmania* infecting man and wild animals in Saudi Arabia. 2. *Leishmania arabica* n. sp. *Trans R Soc Trop Med Hyg 80:* 497–502.
21. Strelkova MV, Shurkhal AV, Kellina OI, Eliseev LN, Evans DA, Peters W, Chapman CJ, Le Blancq SM, van Eys GJ, 1990. A new species of *Leishmania* isolated from the great gerbil *Rhombomys opimus. Parasitology 101:* 327–335.
22. Williams JG, Hanafey MK, Rafalski JA, Tingey SV, 1993. Genetic analysis using random amplified polymorphic DNA markers. *Methods Enzymol 218:* 704–740.