Diagnostic Microbiology and Infectious Disease 77 (2013) 245–247

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Parasitology

*Hind*II and *Sdu*I digests of heat-shock protein 70 PCR for *Leishmania* typing

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ARTICLE INFO

Article history:
Received 19 April 2013
Received in revised form 30 July 2013
Accepted 31 July 2013
Available online 17 September 2013

Keywords:
Leishmania
Typing
*hsp*70
PCR-RFLP

ABSTRACT

Restriction fragment length polymorphisms of the heat-shock protein 70 gene have been used for discriminating *Leishmania* species. Here, we validated *Hind*II as a much cheaper alternative to *Eco*RII and *Sdu*I for discriminating *Leishmania* (*Viannia*) *braziliensis* from *Leishmania* (*Viannia*) *naiffi* and an atypical *Leishmania* (*V.*) *braziliensis* group, which was previously not possible.

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Identification of *Leishmania* is crucial for selecting the most appropriate therapeutic regimen to be administered to each individual, to determine possible control measures in epidemiological studies and to predict the risk of dissemination in immunocompromised patients (Khosravi et al., 2012). Molecular methods are increasingly employed for diagnosis, clinical, and epidemiological studies on leishmaniasis in an effort to detect infection and categorize *Leishmania* at the genus, species, or strain level (Reithinger and Dujardin, 2007; Schönian et al., 2008; Van der Auwera et al., 2013). The cytoplasmic heat-shock protein 70 gene (*hsp*70) has been exploited for *Leishmania* species identification in the Old and New World using the polymerase chain reaction (PCR), followed by sequencing or restriction fragment length polymorphism (RFLP) analysis (da Graça et al., 2012; Fraga et al., 2012; García et al., 2004; Montalvo et al., 2010; 2012; Van der Auwera et al., 2013; Veland et al., 2012). Recently, 3 sensitive and specific PCRs (PCR-F, PCR-N, and PCR-C) and their corresponding RFLPs (RFLP-F, RFLP-N, and RFLP-C) were developed for this target, facilitating its use for both *Leishmania* detection and identification (Montalvo et al., 2012). The described RFLP-N typing scheme uses the restriction endonuclease *Eco*RII for discrimination both in the New and Old World, but it is expensive because 20U are needed for complete digestion of the PCR product (Montalvo et al., 2012), which costs 16.4 € per reaction, and sometimes, incomplete digests are obtained. Moreover, the published typing schemes permit differentiating *Leishmania* (*Viannia*) *braziliensis* from *Leishmania* (*Viannia*) *peruviana* using the restriction enzyme *Rsa*I but do not allow consistent separation of *L.* (*V.*) *braziliensis* from *Leishmania* (*Viannia*) *naiffi*. The differentiation of *L.* (*V.*) *naiffi* is important because this species is less pathogenic to humans than are other species of the *Viannia* subgenus, probably due to its lower infection index (Azpurua et al., 2010; Darie et al., 1996; Pratlong et al., 2002; Rotureau et al., 2006; van Thiel et al., 2010). In addition, current typing schemes do not differentiate *L.* (*V.*) *braziliensis* from an atypical *L.* (*V.*) *braziliensis* group recently described by Odiwuor et al. (2012).

An alignment of 49 *Leishmania hsp*70 gene sequences representing 12 New and Old World human *Leishmania* species (Table 1, Table ESM1) was used to identify suitable restriction endonucleases to remedy these drawbacks. Theoretical fragment sizes and gel patterns were determined with the online tools NEBcutter (http://tools.neb.com/NEBcutter2) and restriction enzyme digest of DNA (http://insilico.ehu.es/restriction/main/index2.php). The following criteria were taken into account for enzyme selection: ability to discriminate the desired species and groups with the fewest possible number of digests, absence of too many small fragments (<50 bp), absence of intraspecific variation, availability, and cost.

The selected enzymes *Sdu*I and *Hind*II were tested on 101 reference strains and isolates (Table 1, Table ESM1). All DNAs were isolated from parasite cultures, and either parasites or DNA was obtained from the various institutes acknowledged at the end of this paper. PCR-N and PCR-F (Montalvo et al., 2012) were used to amplify the *hsp*70 genes, from 10 ng *Leishmania* DNA. A negative control tube containing all the components except DNA was always included. Ten microliters of the PCR amplicons were analyzed on a 2% agarose gel. PCRs were considered positive if a specific amplicon of the expected size (PCR-F 1286 bp, PCR-N 593 bp; Montalvo et al., 2012) was observed. The amplicons were digested in 10 μL containing 1× optimal buffer recommended by the manufacturer, 5 μL unpurified PCR product, and 2U *Sdu*I, 2U *Hind*II, or 20U *Eco*RII (MBI Fermentas, St. Leon-Rot, Germany). Reactions were incubated 3 h at 37 °C (*Eco*RII

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http://dx.doi.org/10.1016/j.diagmicrobio.2013.07.023

Table 1
Strains and isolates.[a]

Species[b]	Country of origin
L. (*L.*) *aethiopica* (n = 8)	6 Ethiopia, 2 Kenya
L. (*L.*) *tropica* (n = 13)	1 India, 1 Iraq, 3 Kenya, 3 Palestinian Territory, 1 Sudan, 2 Namibia, 2 Israel
L. (*L.*) *donovani* (n = 12)	2 Ethiopia, 3 India, 3 Kenya, 4 Sudan
L. (*L.*) *infantum* (n = 23)	5 Brazil, 1 China, 5 France, 2 Italy, 1 Malta, 1 Morocco, 1 Portugal, 7 Spain
L. (*L.*) *major* (n = 10)	1 Burkina Faso, 2 Israel, 1 Kenya, 1 Saudi Arabia, 1 Spain, 2 Sudan, 1 Tunisia, 1 Uzbekistan
L. (*L.*) *amazonensis* (n = 4)	3 Brazil, 1 Peru
L. (*L.*) *mexicana* (n = 2)	1 Belize, 1 Guatemala
L. (*L.*) *garnhami* (n = 1)	1 Venezuela
L. (*V.*) *braziliensis* (n = 19)	8 Bolivia, 4 Brazil, 7 Peru
L. (*V.*) *braziliensis* atypical (n = 3)	2 Bolivia, 1 Peru
L. (*V.*) *guyanensis* (n = 13)	5 Brazil, 1 French Guiana, 7 Peru
L. (*V.*) *panamensis* (n = 2)	2 Panama
L. (*V.*) *naiffi* (n = 4)	2 Brazil, 1 French Guiana, 1 Unknown

[a] A detailed listing is provided in Table ESM-1, with an indication of which 101 isolates were analyzed in RFLP.
[b] Species designations according to current literature. *L.* (*V.*) *braziliensis* atypical is a group of isolates identified by Odiwuor et al. (2012) that are clustered using the analysis of amplified fragment length polymorphisms and sequencing of a heat-shock protein 70 gene fragment, different from the main *L.* (*V.*) *braziliensis* group. The total number of isolates is shown between brackets.

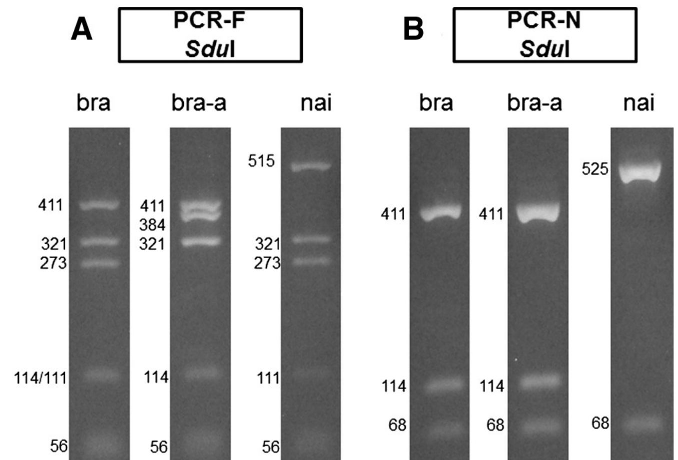

Fig. 2. RFLP patterns using the *Sdu*I restriction endonuclease for amplicons of PCR-F (A) and PCR-N (B). Size indication in base pairs is depicted on the left of each gel lane. *Leishmania* species abbreviations: bra = *L.* (*V.*) *braziliensis*, bra-a = atypical *L.* (*V.*) *braziliensis*, nai = *L.* (*V.*) *naiffi*. In *in silico* analysis, the restriction patterns of *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* are the same. The size of the PCR-F product is 1286 bp, and that of PCR-N, 593 bp.

overnight), and fragments were analyzed in a 3% small fragment agarose gel (Gentaur, Brussels, Belgium), running at 3.5 V/cm for 3 h.

In RFLP-N, *Hin*dII was able to separate the same species groups as does *Eco*RII (Fig. 1), thereby corroborating the results from *in silico* analysis. No intra-species differences were observed in any of the RFLPs. The protocol of Montalvo et al. (2012) uses 20U *Eco*RII per reaction, which amounts to 16.4 €, while the *Hin*dII used here costs 0.20 Euro cents per reaction. In RFLP-N and RFLP-F, *Sdu*I could separate at low cost (0.26 Euro cents) *L.* (*V.*) *braziliensis* from *L.* (*V.*) *naiffi*, in line with the *in silico* analysis (Fig. 2). In addition, this enzyme was able to discriminate the genetic variability among *L.* (*V.*) *braziliensis* described by Odiwuor et al. (2012), with amplicons obtained in PCR-F. All isolates tested showed the respective patterns. According to the *in silico* analysis *L.* (*V.*) *peruviana* produces the same restriction pattern as *L.* (*V.*) *braziliensis*. For the differentiation of these species using PCR-F or PCR-N, the restriction endonuclease *Rsa*I can be used (Montalvo et al., 2012).

L. (*V.*) *naiffi* is a common parasite of armadillos and could be isolated from humans with cutaneous leishmaniasis mostly in Brazil (Grimaldi et al., 1991; Lainson and Shaw, 1989). Nevertheless, the design of molecular tools that allow separation of *L.* (*V.*) *naiffi* from other *Leishmania* species is important in other countries as well. Indeed, host and vector are present in a wide geographical area, and *L.* (*V.*) *naiffi* is probably widespread in South America (Pratlong et al., 2002). Other human cases have been described in patients from Martinique, Guadeloupe, French Guyana, Ecuador, Peru, and Surinam (Darie et al., 1996; Pratlong et al., 2002; Rotureau et al., 2006; van Thiel et al., 2010). Also, the parasite has been reported in sandflies from Panama (Azpurua et al., 2010).

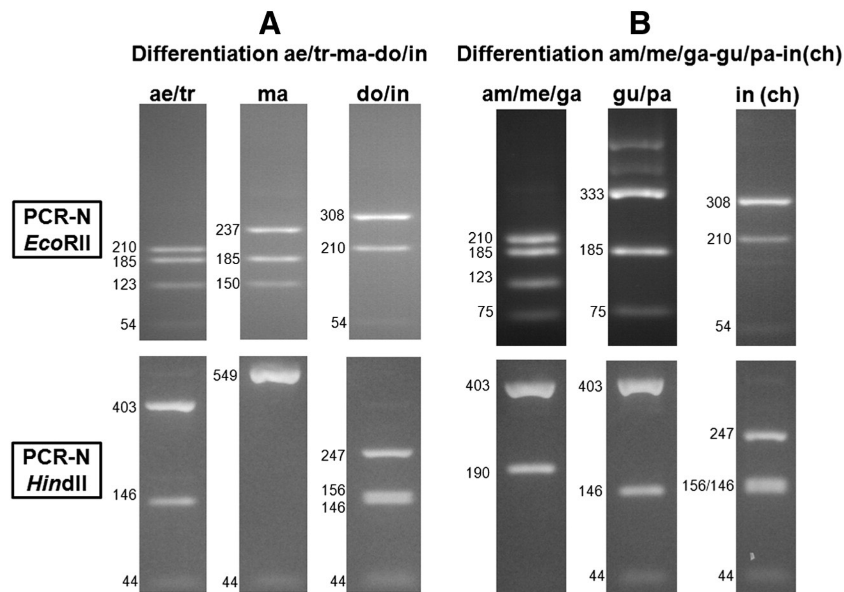

Fig. 1. The RFLP agarose gel patterns using *Eco*RII (Montalvo et al., 2012) and *Hin*dII for the differentiation of *L.* (*L.*) *aethiopica*/*L.* (*L.*) *tropica*, *L.* (*L.*) *major*, and *L.* (*L.*) *donovani*/*L.* (*L.*) *infantum* (A); and *L.* (*L.*) *amazonensis*/*L.* (*L.*) *mexicana*/*L.* (*L.*) *garnhami*, *L.* (*V.*) *guyanensis*/*L.* (*V.*) *panamensis*, and *L.* (*L.*) *infantum* (*chagasi*) (B). Size indication in base pairs is depicted on the left of each gel lane. *Leishmania* species abbreviations: ae = *L.* (*L.*) *aethiopica*, tr = *L.* (*L.*) *tropica*, ma = *L.* (*L.*) *major*, do = *L.* (*L.*) *donovani*, in = *L.* (*L.*) *infantum*, in(ch) = *L.* (*L.*) *infantum* (*chagasi*), am = *L.* (*L.*) *amazonensis*, me = *L.* (*L.*) *mexicana*; ga = *L.* (*L.*) *garnhami*, gu = *L.* (*V.*) *guyanensis*, pa = *L.* (*V.*) *panamensis*. The size of PCR-N product is 593 bp.

The atypical *L.* (*V.*) *braziliensis* group was described by Odiwuor et al. (2012) on the basis of amplified fragment length polymorphism and sequencing of an *hsp*70 gene fragment. The group seems widespread in Latin America, as evidenced by its presence in at least Peru, Bolivia, and Panama. The authors highlighted the need for assays to discriminate these atypical strains from the main *L.* (*V.*) *braziliensis*/*L.* (*V.*) *peruviana* cluster because the group may behave differently from a clinical perspective. Parasites from both *L.* (*V.*) *braziliensis* groups have, nevertheless, been isolated from mucosal lesions (data not shown). As the atypical strains are not recognized in currently deployed assays, no detailed clinical or accurate epidemiological information is available yet. This can only be obtained when appropriated tools, such as the one here described, are applied.

In summary, in this paper, we update the protocols RFLP-F and RFLP-N designed by Montalvo et al. (2012) with new restriction enzymes. These new enzymes reduce cost and allow better separation of some New World (sub)species.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.diagmicrobio.2013.07.023.

Acknowledgments

The authors would like to thank all colleagues and institutes who kindly donated the *Leishmania* reference strains or DNA, among whom J. Arévalo (Instituto de Medicina Tropical Alexander von Humboldt, Lima, Peru); L. García (Centro Universitario de Medicina Tropical, Cochabamba, Bolivia); E. Cupolillo (Instituto Oswaldo Cruz, Rio de Janeiro, Brazil); G. Schönian (Institut fur Mikrobiologie und Hygiene, Berlin, Germany); I. Mauricio and D. Evans (London School of Hygiene and Tropical Medicine, London, UK); P. Desjeux (Instituto Boliviano de Biologia de Altura, La Paz, Bolivia); J.P. Dedet and J.A. Rioux (Centre National de Référence des *Leishmania*, Montpellier, France); J.J. Shaw (University of Sao Paulo, Sao Paulo, Brazil); G. Schoone and A. El Harith (Royal Tropical Institute, Amsterdam, The Netherlands); I. Felger (Swiss Tropical and Public Health Institute, Switzerland); and the Leishepinet consortium (EU contract INCO-CT2005-015407). This work has been funded in part by the third framework program of the Belgian Directorate General for Development with ITM Antwerp.

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