INTRODUCTION

Use of species-specific recombinant DNA probes opened new insights in the field of eco-epidemiological investigations of leishmaniases mainly because of the identification of the Leishmania sp. organisms concomitantly to their detection \(^1,2\). Classically, Leishmania identification requires parasite isolation and growth into mass-culture; species attribution comes in a second step referring either to isoenzymes \(^3,4\) and/or to genomic DNA probes \(^5,6,7\). Another interest in specific DNA probes could be identification of pro-

RESUME

Des sondes ADN spécifiques du parasite Leishmania (L.) infantum ont été analysées selon différentes stratégies utilisant des minicercles recombinants isolés à partir de l’ADN kinétoplastique de L. infantum. Une première sonde a été identifiée à la suite d’une procédure classique. Un minicercle sélectionné pour sa forte réactivité vis-à-vis de l’ADN total de L. infantum a été utilisé pour l’identification de fragments spécifiques de cette espèce, dont un de 95 pb 3B8HaeIII-2, a été sélectionné. Pour l’obtention de la seconde sonde, une stratégie basée sur un criblage différentiel ciblant la sensibilité et la spécificité a été utilisée. Ceci a permis d’identifier un groupe de minicercles. Des sous-clonages ainsi que des criblages différentiels ont conduit à la sélection d’un fragment de 137 pb, 3E9HaeIII-12. Les réactivités des deux sondes ont ensuite été évaluées à la suite de l’application de ces dernières à une gamme d’ADN totaux et de promastigotes issus de 74 isolats appartenant à 9 espèces rencontrées dans l’Ancien Monde. Des parasites isolés en Tunisie dans différents foyers, à partir de différents hôtes après différentes saisons de transmissions, ont été inclus dans cette gamme. Les résultats des hybridations ont montré une spécificité exclusive de ces deux sondes pour L. infantum dans ce pays. La sonde 3E9HaeIII-12 s’est avérée plus sensible puisque 10 ng d’ADN total et 10\(^3\) promastigotes peuvent être détectés. À la lumière de ces résultats et en comparaison avec les données de la littérature, la seconde procédure a permis une augmentation de la sensibilité d’au moins dix fois.

Mots clés: L. infantum, minicercle, sondes ADN spécifiques, spécificité, diagnostic, Leishmaniose,

ABSTRACT

The study refers to the isolation of specific DNA probes to the parasite species Leishmania (L.) infantum according to different strategies using recombinant minicircles isolated from L. infantum kinetoplast DNAs. A first probe was identified following a classical procedure. One minicircle selected for strong reactivity to L. infantum total DNA was used to identify specific subfragments to this species among which the 95bp fragment, 3B8HaeIII-2 was selected. For the obtention of the second probe, a strategy based on sequential screenings for specificity and sensitivity was applied. This allowed identification of a set of minicircles showing an increased specificity to L. infantum as compared to other species, and an increased sensitivity of reaction as compared to the other minicircles. Subclonings and screenings allowed a final selection of a 137bp - minicircle fragment: 3E9HaeIII-12. Reactivities of the 2 probes were assessed on a panel of total DNAs and promastigotes from 74 isolates pertaining to 9 species encountered in the Old World. Parasites isolated in Tunisia from different foci, different hosts after different transmission seasons were included. Hybridizations have shown the exquisite specificity of these probes to L. infantum in this country. Probe 3E9HaeIII-12 was found to be the more sensitive where down to 10 ng of total DNA and 10\(^3\) promastigotes could be detected. From this study and as compared to data provided in the literature, the second procedure allowed at least 10-fold increase in sensitivity.

Key Words: L. infantum, minicircle, specific DNA probe, sensitivity, diagnosis, leishmaniases,

INTRODUCTION

Use of species-specific recombinant DNA probes opened new insights in the field of eco-epidemiological investigations of leishmaniases mainly because of the identification of the Leishmania sp. organisms concomitantly to their detection \(^1,2\). Classically, Leish-
mastigotes soon after their isolation, which would require lesser parasites than classical methods. Specific DNA probes aimed for diagnostic purposes need to be sensitive, therefore targeting repeated DNA sequences. Leishmania kinetoplast minicircles constitute candidates of choice because of their unique presence among Kinetoplastida and relative abundance. Furthermore, they were shown to bear specificities to taxa, species and even isolates and were successfully targeted to develop specific DNA probes for Old World Leishmania species. Two ways were considered: the classical approach of selecting specific subfragments from a given minicircle. The second one took into account the description of different minicircle classes and the existence of representational variabilities among them. Therefore, criteria of sensitivity was considered in addition to specificity for the screenings which were done on a collection of 56 cloned minicircles obtained from three independent isolates. Each procedure allowed to identify a probe, 3B8HaeIII-2 & 3E9HaeIII-12, respectively. The reactivity of these probes was assessed and compared on a panel of dot-blotted-promastigotes and -total DNAs from different Leishmania species. Three criteria were tested. Specificity of the probes was checked on a panel of representatives of the different Old World species. Consistency of the reactivities (positive or negative) in Tunisia was assessed whenever possible, among samples of the same species obtained from different foci, hosts and/or vectors and isolated after different transmission seasons. Sensitivity of reaction was tested among a panel of Tunisian L infantum isolates. Probes were found to be equally specific but 3E9HaeIII-12 was found to be more sensitive than 3B8HaeIII-2. Therefore, probe 3E9HaeIII-12 appeared as a specific and sensitive kDNA fragment, which would allow diagnosis of L infantum in Tunisia. Furthermore, from this work and as compared to data described in the literature, the procedure devised allowed a gain of at least 10-fold in the sensitivity of reaction.

**MATERIAL AND METHODS**

**Parasites:** They are listed on Table 1. Seventy five isolates were used, corresponding to at least 43 zymodemes, representatives of 10 species: L infantum, L chagasi, L donovani, L major, L tropica, L killicki, L turanica, L arabica, L aethiopica and Leishmania tarentolae. The parasites were obtained from various hosts and vectors in 12 countries. The selection of Tunisian isolates is well representative of the parasites available, originating from the different transmission foci. Species identification was confirmed as previously described.

**Minicircles:** They were isolated from three stocks of in vitro promastigotes of Tunisian L infantum from visceral leishmaniasis patients (IPT1 and Moalla) and from an infected dog (CN64). Minicircles were cloned in plasmid pUC19 after ligation of EcoRI or BamHI fragments purified from gels as in van Eys et al. (1989). A total of 56 recombinants were kept for further study on the basis of their size and high reactivity with IPT1-total DNA probe and with a 17-mer oligonucleotide probe, universal to Kinetoplastida, selected from data banks.

**DNA probes:** Total DNA probes from L infantum (IPT1, Moalla, CN64), L major (TN435) and L killicki (Tat534), were used for the screening of the minicircles present at a high copy number. Preferential reactivities for L infantum were assessed from the signals. To test and compare sensitivity of the minicircles selected, they were used to probe blotted Leishmania - total DNAs.

**DNA techniques:** Total DNA was obtained from mass cultures of promastigotes as previously described. Plasmid purification and cloning followed classical procedure. Restriction and modification enzymes were used according to manufacturer instructions (Boehringer, Germany and Pharmacia, Sweden). The probes were radioactively labelled with α-PdCTP (3000 Ci/mmol, Amersham, France). Minicircles and subclones were sequenced on both strands using T7 DNA polymerase (Pharmacia, Sweden), α-S-32 PdATP (>1000 Ci/mmol, Amersham, France) and the M13-Universal and Reverse primers as well as internal ones.

**Subclonings:** The purified inserts were digested with Hhal and HaeIII. The products were repurified and subcloned into Smal site of plasmid pUC19. After transformation, the white colonies were picked on nitrocellulose filters in triplicate and screened differentially using total DNA probes. Subclones showing specific reactivity with L infantum were screened for sensitivity by probing serial amounts of in vitro promastigotes.

**Dot-blot:** Total DNAs were denatured in 0.1N NaOH, neutralized in 0.15M NaH2PO4 and dot-blotted onto Hybond N membranes (Amersham, France). For the promastigotes, following the blotting, the membranes were treated successively in 1N NaOH, 1M Tris-HCl and (0.5MTris-HCl / 1.5M NaCl). The membranes were air-dried and baked at 80°C for 2 hours. Hybridizations were done according to Guizani et al. (1994). Membranes were exposed to X-ray films (X-OMATK, Kodak) at -80°C using enhancer screens during two and six nights.

**Estimate of the sensitivity of reaction:** The sensitivity of reaction (S) is defined by “S = P / N ”, where P and N correspond, respectively, to the number of positive responses obtained with the L infantum isolates and to the total number of the L infantum isolates tested. The sensitivity was expressed for each amount of DNA or promastigotes tested.
RESULTS
Selection of a specific DNA probe for the parasite species *L. infantum*: The work aimed at identifying a specific minicircle fragment to the species *L. infantum*. To this purpose, it was decided to identify a restriction fragment that bears specificity to *L. infantum* starting from an abundant minicircle. Such a minicircle, 3B8 was selected from a collection of recombinant molecules based on strong reaction to a *L. infantum* total DNA probe. HaeIII subclones were differentially screened using total DNA probes for the species *L. infantum*, *L. major* and *L. killicki*. This allowed to select 6 subclones among which 3B8HaeIII-2 proved to be the most sensitive (Figure 1). However, application of this probe to a panel of DNAs or promastigotes soon proved the limits in the usage of this probe as regards to its sensitivity of reaction (Figure 2C). This leaded us to explore other ways in identifying specific and sensitive minicircle fragments.

Selection of minicircles bearing sequence specificity to *L. infantum* and high sensitivity of reaction: Here, the existence of different minicircle classes represented in variable copy number according to the isolates was taken into account. Therefore, criteria of specificity and sensitivity were considered during all screening procedures. Total DNA probes for the species *L. infantum*, *L. major* and *L. killicki*, encountered in Tunisia, were applied to a collection of dot-blotted minicircles. All minicircles hybridized to the 3 *L. infantum* total DNAs, purified from the isolates considered to clone the minicircles. However, the intensity of the positive signals was variable and did not depend on the isolates. Furthermore, the inserts cross-reacted to a limited extent with the two other species. Nevertheless, the signal was always lower with *L. killicki*. On the basis of strong reactivity to the 3 *L. infantum* total DNA probes and poor cross hybridization with other species, six minicircles (1E1, 1E9, 1E12, 3E9, 3E20 and 3E26) were selected. Subsequently, they were used to probe decreasing dot-blotted amounts of *Leishmania* - total DNAs or - promastigotes from representatives of the 3 species met in Tunisia, thus screening for the probes expressing the highest sensitivity of reaction. Probes 1E1 and 3E9 were able to react clearly down to 1ng *L. infantum* DNA while the other four only reacted down to 10ng DNA. Similar results were obtained with dot-blotted promastigotes. Minicircles, 1E1 and 3E9 were therefore selected for further screening.

Screenings for specific and sensitive minicircle fragments to *L. infantum*: Here, it was aimed to refine the specificity of reaction of the minicircles selected. The inserts were purified, digested with HhaI and HaeIII and subcloned. The subclones were screened differentially as before. The four recombinants exhibiting a specific and strong signal with *L. infantum* total DNA were selected and used to probe dot-blotted *L. infantum* (IPT1) and *L. major* (TN435) promastigotes (Figure 1). The results confirmed the specificity of all fragments toward *L. infantum*. However, as regards to the sensitivity, only one probe,

![Figure 1: Screening of the minicircle subclones according to sensitivity. Decreasing amounts of in vitro *L. infantum* (IPT1, a) and *L. major* (TN435, b) promastigotes, ranging from 10⁶ to 10³, were dot-blotted onto nylon membranes, denatured and probed with the subclones of 1E1, 3E9 and 3B8 selected in a previous step for specificity. This allowed the selection of the 2 probes 3E9HaeIII-12 and 3B8HaeIII-2.](image-url)
3E9HaeIII-12, reacted strongly down to 10^4 promastigotes of *L. infantum*. Finally, the two minicircles fragments, 3E9HaeIII-12 & 3B8HaeIII-2 were kept for the rest of the study and submitted to molecular characterization and evaluation. DNA sequencing allowed mapping these fragments precisely on the parental minicircles. They correspond respectively to a 137 bp-HaeIII and a 95 bp-BamHI-HaeIII-fragments, located in the variable region 340 bp and 385 bp downstream the conserved region. They have a GC content of 47% and 50%, respectively. Minicircles 3E9 and 3B8 belong to 2 minicircle sequence classes. From hybridization results, 3E9 is represented differently than 3B8.

Assessment and compared evaluation of the reactivity of the probes 3E9HaeIII-12 and 3B8HaeIII-2: To check for the reactivity of the probes and their specificity, the DNA recombinants were applied to a panel of *Leishmania* isolates of different geographical origins, representatives of different species. Consistency of the reactivities was assessed among parasites of the same species obtained from different foci, hosts and vectors and isolated during different transmission seasons. Probes were tested on serial amounts of total DNAs and promastigotes ranging from 1µg to 1ng and 10^6 to 10^3, respectively (Table 1). Part of the results observed is illustrated on Figure 2A. Both 3E9HaeIII-12 and 3B8HaeIII-2 revealed strong hybridizations with *L. infantum* and *L. chagasi* parasite DNAs. Weaker cross reactivities were observed with *LEISHMANIA INFANTUM* SPECIES-SPECIFIC KDNA PROBES: ISOLATION AND EVALUATION

Table 1: Selection of the Leishmania parasites used in this study.

The table summarizes information on the material used in this study (DNA or promastigotes) for each isolate. WHO codes and species attribution are also provided. The species identification was confirmed on the DNA preparations using DNA probes exerting species-specific hybridization patterns, save for the *L. tarentolae* isolate (**) for which we relied on the donor (Istituto Superiore di Sanità, Rome, Italy). Information on zymodeme type was given by donors. T: tested. NT: not tested.
L. donovani at $10^6$. No reaction was observed with the parasites belonging to the other species tested: L. major, L. tropica, L. killicki, L. aethiopica, L. arabica, L. turanica and L. tarentolae with regards to all the amounts tested. Longer exposure did not reveal any additional cross reactivity (not shown). At the highest amounts tested (1µg to 100 ng), all L. infantum DNAs reacted with the probes. The signal intensity then decreased and was variable from an isolate to another. In total, the DNA probes proved to exert an exquisite specificity to L. infantum in Tunisia.

As regards to the sensitivity of reaction, probe 3E9HaeIII-12 showed to be more sensitive than 3B8HaeIII-2 for detection of 10ng of total DNA. This parameter was further estimated on promastigote counts. Probe 3E9HaeIII-12 could detect down to $10^3$ promastigotes (figure 2B). Table 2 summarises the measures of the sensitivity of reaction for each amount tested. With $10^6$ to $10^3$ promastigotes, the technique was found 100% sensitive with 3E9HaeIII-12. However, using probe 3B8HaeIII-2, 100% sensitivity is only observed for $10^6$ promastigotes.

**DISCUSSION**

The work aimed at the isolation of specific and sensitive minicircle fragments for diagnostic purposes of L. infantum in Tunisia. Species-specific minicircle fragments for Leishmania parasites were mainly identified after differential screening of restriction digests from total kDNA, or few cloned minicircles. Here, alternatively, in another approach, we took into account the existence of different minicircle classes in variable copy numbers. Collections of recombinant minicircles originating from three independent L. infantum isolates were screened.

**Table 2:** Sensitivities of reaction for the detection of L. infantum parasites isolated in Tunisia. They are reported for each probe according to the amounts tested of promastigotes (106 to 103) or total DNA (1mg to 1ng). The sensitivity corresponds to the number of isolates that reacted positively by the total number of isolates assessed.

<table>
<thead>
<tr>
<th>Sensitivity</th>
<th>Promastigote amounts</th>
<th>DNA amounts</th>
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<tbody>
<tr>
<td></td>
<td>$10^6$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>Probe 3E9 HaeIII-12</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Probe 3B8 HaeIII-2</td>
<td>100%</td>
<td>86%</td>
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isolates were subjected to stringent sequential screenings in order to fulfill criteria of specificity and sensitivity, respectively. As shown here, this approach proved its efficiency in identifying sensitive DNA sequences. Two probes were selected during this work, 3E9HaeIII-12 and 3B8HaeIII-2, which were assessed on a panel of 74 isolates. Only one study used a comparable number (N=73) of isolates. Representatives of most parasite species encountered in the Old World were included in the panel. Whenever possible, it was attempted to include parasites originating from different loci, hosts or vectors, isolated during different transmission seasons. Emphasis was made on L major (n=27) and L tropica complex (n=19) which are also endemic in Tunisia. The probes did not react with these isolates or with reference isolates for the species L. arabica, L. turcica, L. ethiopica and L. tarentolae all isolates of the L donovani species-complex reacted positively; intensities being stronger with L. infantum. The cross-reactivity to L. chagasi (syn. L. infantum) and L. donovani could be expected as very few DNA probes allowed clear cut distinctions among L infantum/chagasi and L. donovani. However, this would not constitute a hindrance to the identification of L. infantum parasites in Tunisia as these species are not met in the West Mediterranean basin. Exquisite specificity of the probes toward L. infantum in Tunisia could thus be concluded. As regards to the sensitivity of reaction, which constituted a major concern of the study, probe 3E9HaeIII-12 globally was shown to be more sensitive than 3B8HaeIII-2, using either DNA or promastigotes as test material. This is to be related to the different screening schemes that were followed for each probe. A survey of the literature on data provided for other kDNA probes, reveals that sensitivity does not seem to have been systematically tested before. However, L major and L. infantum kDNA probes showed positive signals for all isolates tested of the target species, with 10^6 and 10^5 promastigotes, respectively; then the number of isolates showing positive signals dropped 11-13. In this report, with 3E9HaeIII-12, the tests showed a score of 100% sensitivity for amounts ranging between 10^6 and 10^4 promastigotes. It dropped to 64% with 10^3 promastigotes. Probe 3B8 HaeIII-2 gave consistently positive signals only with 10^4 parasites. The screening schemes devised allowed a gain of at least 10-fold in the sensitivity of reactions. The results suggested a variability in the representativity (copy number and/or homology) of the probed sequences in the different parasites assayed which, in spite of the abundance of the minicircles, seemed to interfere with the sensitivities of the reactions of the probes. This could be related to the existence of a polynality in the specificity of sequences born by the variable region 11-13, also confirmed here for L. infantum. Indeed, sequence analysis located the kDNA probe fragments under study on the variable region, at similar locations downstream the conserved region. Therefore, this also emphasizes the existence of dominant classes of minicircles among which highly sensitive sequences could be identified, eg. 3E9HaeIII-12 as compared to 3B8HaeIII-2. Mechanisms proposed for generation of minicircle diversity could also account for the probes limitations in terms of sensitivity 3. To overcome limits related to minicircle variability, one could tempt to refer to nuclear repeats. However, highly repeated sequences remain prone to copy number variations. Specific genomic repeats to the L. donovani complex were identified which could detect 100 parasites. However, the study did not illustrate consistent reactivity with all parasites assayed, at this level of detection 13. In total, the identification of diagnostic probes for Leishmania parasites necessitates trade-offs between specificity, sensitivity of reaction and consistent reactivity within a species. Consequently, this controls the conditions necessary for the screening procedures. An increase in sensitivity was achieved in this study. Consistent reactivity is an essential criterion to consider, as it affects epidemiological parameters of the tests when using field samples. Such parameters allow appropriate result interpretations in using the probes for the diagnosis of infections. The actual aim of developing species-specific DNA probes was their application to a relatively rapid detection, concomitant to the identification of promastigotes or amastigotes. This has the advantage of processing a large number of samples together. Further work is in progress to assess their appropriate use for the diagnosis of Leishmania infections. However, this study allow to recommend use of these probes for the rapid identification of well-defined amounts of L. infantum promastigotes spotted on nylon membranes, soon after their isolation, where at least 10^3 parasites can be made available. **AKNOWLEDGEMENTS** The authors are grateful to Dr. G. Van Eys (Maastricht) for discussions and Dr. D. C. Barker for critical comments on the manuscript. This work enabled ABH to defend a "DEA de génétique et biologie moléculaire" at the University of Tunis. This work has received financial support from the UNDP / World Bank / WHO special programme for Research and Training in Tropical Diseases (TD/RSG Grant: ID 890266), the CEC-DGXII - STD3 program (TS3.CT.930253) and MERC- NIAID - NIH (NO1AI 45183). **BIBLIOGRAPHY** 1- D. F. Wirth, and McMahon-Pratt D. (1982). Rapid identification of Leishmania species by specific hybridization of kinetoplast DNA in cutaneous lesions. Proc. Natl. Acad. Sci. USA. 79, 6999 - 7003