Use of Molecular Probes and PCR for Detection and Typing of *Leishmania* - a Mini-Review

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The use of molecular tools to detect and type Leishmania *species in humans, reservoirs or sandflies has been pursued using different approaches. The polymerase chain reaction provided sensitivity to case this task, since the use of hybridization procedures alone employing specific probes is hampered due to the low detection limit. In this report, we describe the different molecular targets used in our laboratory, aiming at the detection and specific typing of these protozoa. Different kits based on hybridization assays and PCR amplification using kinetoplast and nuclear targets are described and the results obtained from their use are reported.*

Key words: *Leishmania* - polymerase chain reaction - hybridization - molecular probes - mini-exon gene - minicircle - kDNA

Clinical manifestations in human leishmaniasis may vary from simple cutaneous lesions to mucocutaneous ulcers or visceral commitment, as a consequence of the complex host immunological response and depending upon the infecting species involved (Grimaldi & Tesh 1993). Traditionally, *Leishmania* is clustered in dermotropic and viscerotropic species, but with the description of cutaneous lesions caused by viscerotropic leishmania and the observation that *L. braziliensis* can cause visceral presentations in patients with the acquired immunodeficiency syndrome, this classification is not strictly supported (Oren et al. 1991, Rioux & Lanotte 1991, Da-Cruz et al. 1992, Hernandez et al. 1993).

The human pathogenic *Leishmania* species from Latin-America belong to two different taxonomic groups : (1) sub-genus *Viannia*, such as *L. braziliensis*, *L. panamensis*, *L. guyanensis*, *L. peruviana* etc., that cause cutaneous or mucocutaneous lesions and (2) sub-genus *Leishmania*, such as *L. mexicana* and *L. amazonensis* that cause localized or diffuse cutaneous involvement and the New World viscerotropic *Leishmania*, *L. chagasi*.

In general, American tegumentar leishmaniasis is characterized by straightforward clinical presentations, but sometimes such aspects can be confused, with difficult differential diagnosis in geographic areas where *Viannia* and *Leishmania* sub-species overlap.

Diagnostic procedures for tegumentar leishmaniasis are based on clinical presentation, epidemiological history, parasitological tests such as microscopic analysis of stained smears (frotis), axenic cultures of biopsy samples or aspirates of lesions, immunological methods such as delayed type hypersensitivity (Montenegro skin tests) and, as a last resort, histopathological analysis with regular staining or using immunoperoxidase. These tests are often time consuming and cumbersome, lack sensitivity or require culture facilities. Furthermore, after the isolation of the species through culture, other methods are necessary to type them.

Since the 80's, several biochemical and immunological methods were developed in an attempt to detect and type *Leishmania* species in clinical samples. The analyses based on enzyme electrophoretic profiles, monoclonal antibodies, kinetoplast DNA (kDNA) restriction digests (schizodeme analysis), specific molecular probes for the nuclear or mitochondrial genome and karyotype profiles have contributed to taxonomic

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and diagnostic studies of these organisms (Lopes et al. 1984, Barker 1987, Ramirez & Guevara 1987, Cupolillo et al. 1994).

Wirth and MacMahon-Pratt (1982) described the absence of cross-hybridization between *L. braziliensis* and *L. mexicana* total kDNA. This discovery opened new insights directed towards the development of molecular probes based on "minicircle" molecules, the major components of the kDNA. These molecules show features that make them almost ideal as molecular targets, since they are present in the kinetoplast network in a high copy number (about 10.000) and contain a conserved region of at least 120 base pairs (bp), that can be evidenced in every molecule. Complete cloned minicircles and fragments were used both for typing and detection of *Leishmania* species with success (Barker & Butcher 1983, Barker et al. 1986, Lopes & Wirth 1986, Rogers et al. 1988), however it was soon recognized that their use in a diagnostic hybridization assay was hampered due to the low detection limit of about 100-1000 parasites per clinical sample, a limitation easily encountered in cutaneous lesions caused by dermotropic *Leishmania (Viannia)* species.

Other probes, derived from different parts of the nuclear genome were developed, but did not bring improvements in the sensitivity. Minicircle sequences and structure were analyzed in more detail and, with the advent of the polymerase chain reaction (PCR) technique (Saiki et al. 1988), very sensitive and promising systems were developed both for detection and subsequent typing of the parasites.

The main applications of PCR for detection and typing of *Leishmania* can be summarized as follows: (a) in diagnosis of leishmaniasis, PCR can provide sensitive and fast detection of *Leishmania* in clinical samples in case of doubt about correct diagnosis, permit detection of infected insect vectors and reservoirs, substitute axenic culturing with a faster, more sensitive method while avoiding population selection in the isolate, substitute for the current biopsy sampling using less invasive techniques such as analysis of lesion aspirates and permit follow-up of treatment through analysis of aspirates of healing lesions, (b) for typing of *Leishmania* isolates in clinical material, insect vectors, reservoirs or cultures and specimens from culture collections, PCR permits schizodeme analysis, using the complete minicircle amplification system and subsequent digestion of the PCR products with restriction enzymes and the use of secondary subgenus-specific typing probes to discriminate between *L. (Viannia)* and *L. (Leishmania)* subgenus, and to identify *Leishmania/Sauroleishmania* genera. Several detection systems based on PCR methodology have been developed and are available. They are either based on kDNA minicircle conserved region or complete circle amplification (Rodgers et al. 1990, De Bruijn & Barker 1992, Smyth et al. 1992, Arevalo et al. 1993), or on amplification of ribosomal RNA, rDNA (Uliana et al. 1991), or nuclear sequences such as the mini-exon repeats (Fernandes et al., in press) or on fingerprinting (Macedo et al. 1992).

In this mini-review, different typing probes based on cloned minicircle fragments or synthetic oligonucleotides, as well as PCR systems developed in our laboratory, aiming at detection and typing of the different *Leishmania* species are reported. These systems are currently being tested with a variety of clinical samples in collaboration with several other laboratories in Brazil and Latin America.

LEISHMANIA DETECTION AND TYPING KITS

"Leishtype" kit - In the Leishtype kit, cloned minicircle molecules from three different *Leishmania* species (*L. panamensis*, *L. amazonensis* and *L. chagasi*) and an oligonucleotide, designed from the analysis of a multiple sequence alignment of the conserved regions of five distinct classes of minicircles from *L. braziliensis* (Fernandes 1992) and further literature data, are provided in order to be used as molecular probes in hybridization assays with radioactive or non-radioactive labeling. The system can be used on a wide variety of samples where sensitivity is not critical. An experimental version of the kit was tested successfully with field samples, permitting to distinguish between the traditional clusters of New World *Leishmania*: dermotropic *Viannia* species, dermotropic *Leishmania* species and the visce-rotropic *L. chagasi*.

"Leishmini-amp" kit - In 1990, Rodgers et al. proposed the use of PCR for the detection of *Leishmania* in clinical samples, amplifying part of the conserved region of kDNA minicircle molecules. Choosing the L- and H-strand replication origins in the *L. amazonensis* mini repeats as oligonucleotide hybridization target sequences, a 120 bp fragments could be amplified, from *L. amazonensis* as well as from other New World *Leishmania* species (Fig. 1). After sequence determination of several other minicircle sequences from different species, and computer analysis of these and literature data, we optimized oligonucleotide sequences in order to reach more universality for the *Leishmania* genus. The system permits the amplification detection in a wide variety of clinical samples with excellent sensitivity (Fig. 2). Subsequent hybridization of the PCR-generated products permits confirmation of the result and typing.

"Leishcircle-amp" kit - As described in the "leishmini-amp" kit, an specific fragment of the

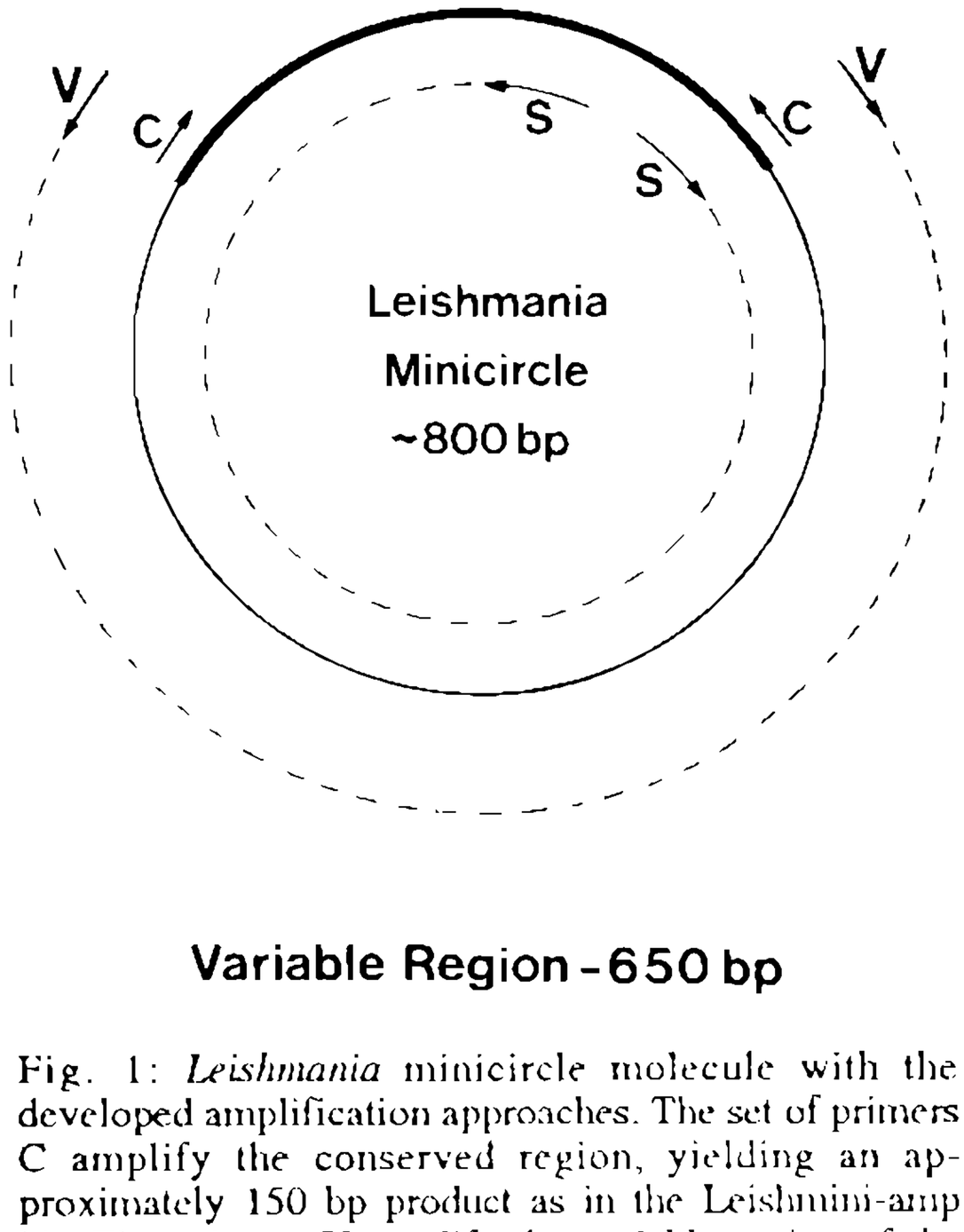

Fig. 1: *Leishmania* minicircle molecule with the developed amplification approaches. The set of primers C amplify the conserved region, yielding an approximately 150 bp product as in the Leishmini-amp kit. The primers V amplify the variable region of the minicircle molecule and such product is useful in typing experiments as in the Leishcircle-amp kit. The primers called S amplify the whole molecule and the product obtained from this PCR is suitable for schizodeme analysis as in the Leishschizo-amp kit.

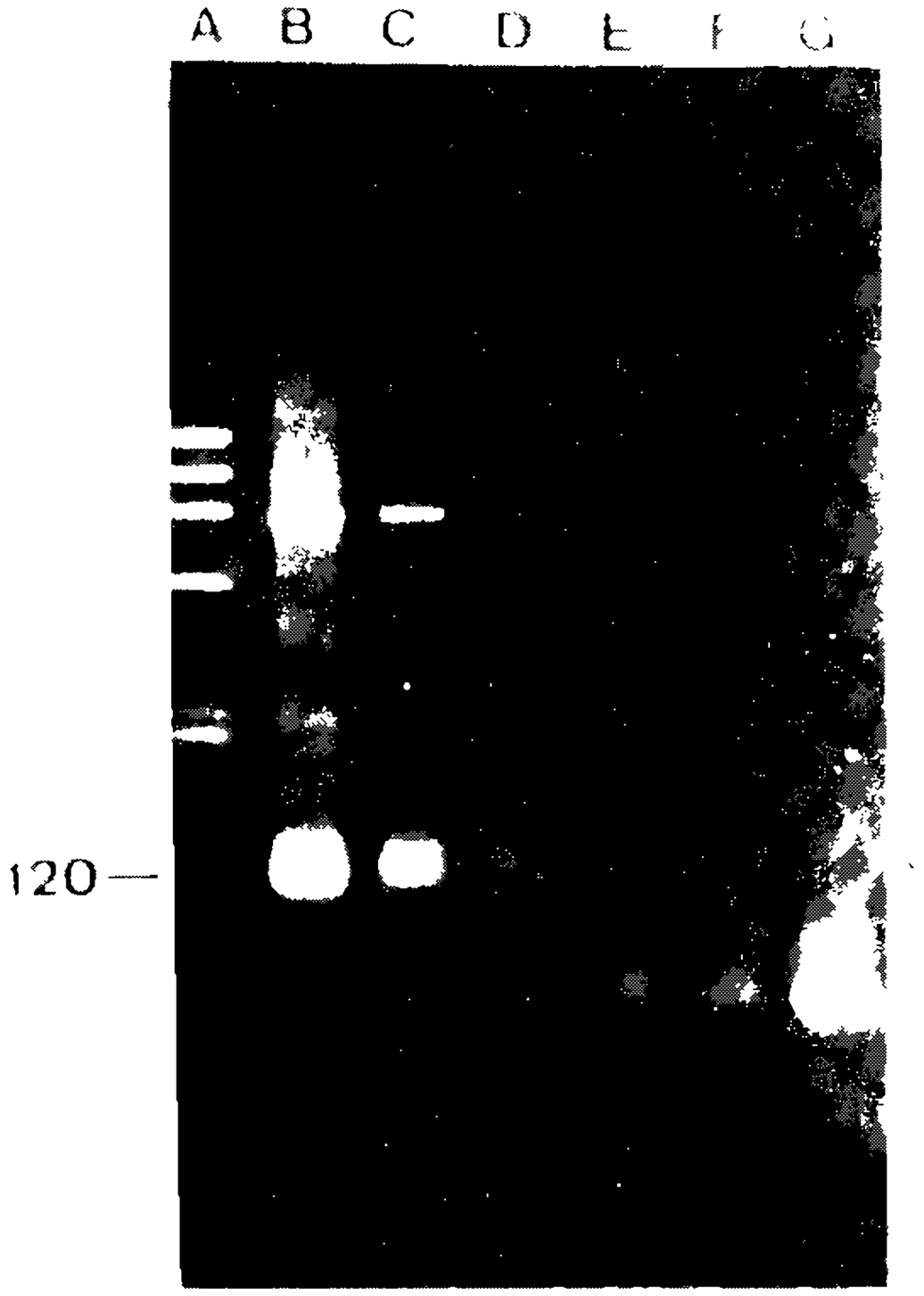

Fig. 2: sensitivity of the PCR system amplifying the conserved region of *Leishmania* minicircle molecules evidenced through agarose gel (3%) electrophoresis of amplified products. Lane A. Molecular marker weight is fx DNA digested with Hae III. The PCR reactions were performed with the following quantities of *Leishmania amazonensis* M2269 kDNA: Lane B. 10ng; Lane C. 1 ng; Lane D. 100 pg; Lane E. 10 pg; Lane F. 1 pg; Lane G. 100 fg.

minicircle conserved region, can be specifically amplified. Likewise, we can amplify the complete variable region of the same target molecule, using the complementary primers of the ones in the aforementioned system. This allows for the generation of 600-700 bp probes, specific for the sequence classes of the minicircles of the originating species or isolate (Fig.1). As the minicircle population within a kDNA network is organized in at least 10 different classes, with respect to the variable region, and as it is expected that different species do not necessarily share such classes, variable region probes could show potential as species specific markers. Further research is needed to explore this possibility.

"Leishschizo-amp" kit - Considering the results described by Lopes et al. (1984), *Leishmania* species can be discriminated by the restriction fragment polymorphism analysis of the minicircle molecule (schizodeme analysis). These different patterns have their origin in the sequence divergence found mainly in the variable region of the different classes of the minicircles in the same network. Using a pair of adjacent primers directed outwards from the conserved region we could amplify (Fig. 1), in the case of *L. Viannia* species, the whole minicircle and submit this product to restriction enzyme digestion, analyzing the products in polyacrylamide gels. A similar approach was used before by Sturm et al. (1989) in order to characterize different *T. cruzi* strains. We expect to use an identical approach for species of the *Leishmania* subgenus, as it was shown by Smyth et al. (1992) that, at least for the viscerotropic species, a specific oligonucleotide sequence, adjacent to the universal 12-mer sequence (the H-strand origin of replication) can also be distinguished, allowing for a "back-to-back" amplification approach.

"Leishexon-amp" kit - The mini-exon gene is present in the nuclear genome of *Kinetoplastidae* in approximately 200 copies, arranged as tandem repeats, and consists of three different parts: an exon, an intron and an intergenic region. The exon is a 39 nucleotides sequence, highly conserved in all *Kinetoplastidae*, which is added

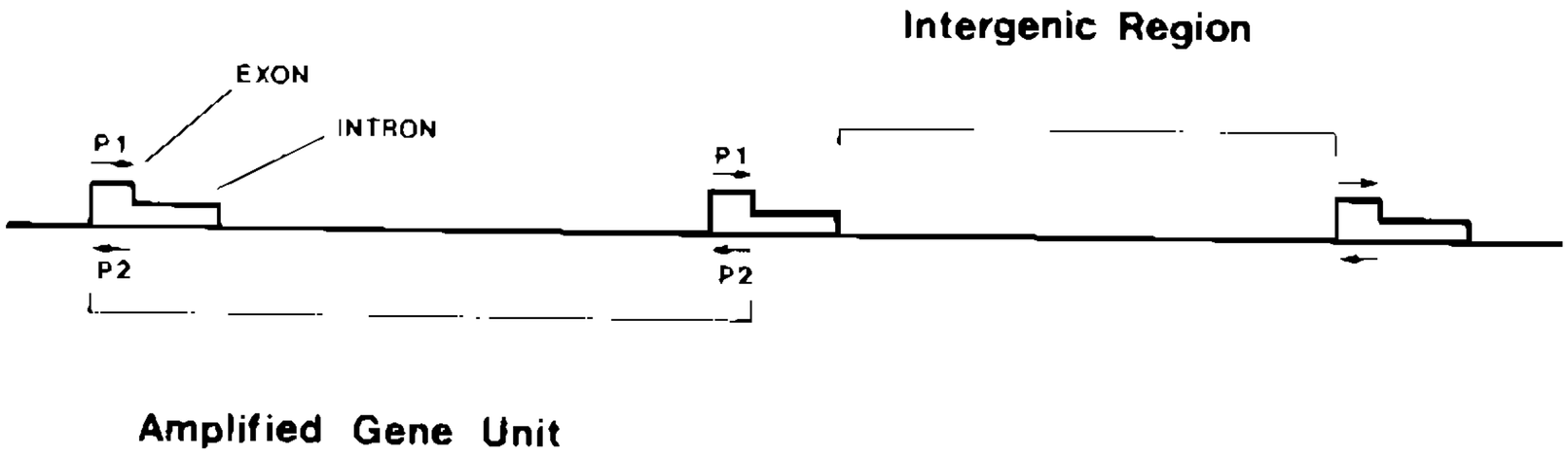

Fig. 3: mini-exon gene amplification scheme. The set of primers used (p1 and p2) is capable of amplifying the complete gene unit as in the Leishexon-amp kit, with its three distinct parts: exon, intron and intergenic region.

post-transcriptionally to the 5'-end of all nuclear messanger RNAs (Campbell et al. 1984). The intron is moderately conserved among the species from the same genus or subgenus, and the intergenic region is very divergent between the different species with respect both to the size and the primary DNA sequence. These characteristics of the intergenic region were used previously to discriminate between the closely related New World *Trypanosoma* species: *T. cruzi* and *T. rangeli* (Murthy et al. 1992), in a mini-exon gene amplification with primers directed to the exon region (Fig. 3). The amplified product was submitted to size analysis in agarose gels and hybridization detection using the intergenic region as a molecular probe.

We investigated and confirmed the potential use of this approach to identify and type *Leishmania* species (Fernandes et al., in press). The mini-exon gene is able to cluster the New World *Leishmania*, based on the gene size and sequence of the intergenic region, in three groups (Fig. 4): *Viannia* species (250 bp), dermotropic *Leishmania* species (350 bp) and *L. chagasi* (400 bp). Although the mini-exon genes are present in many copies in the nuclear genome, a "Leishexon-amp" kit is mainly destined for typing and identification of species, and less for diagnostic detection purposes, since the sensitivity of the amplification system seems inferior to the minicircle approach.

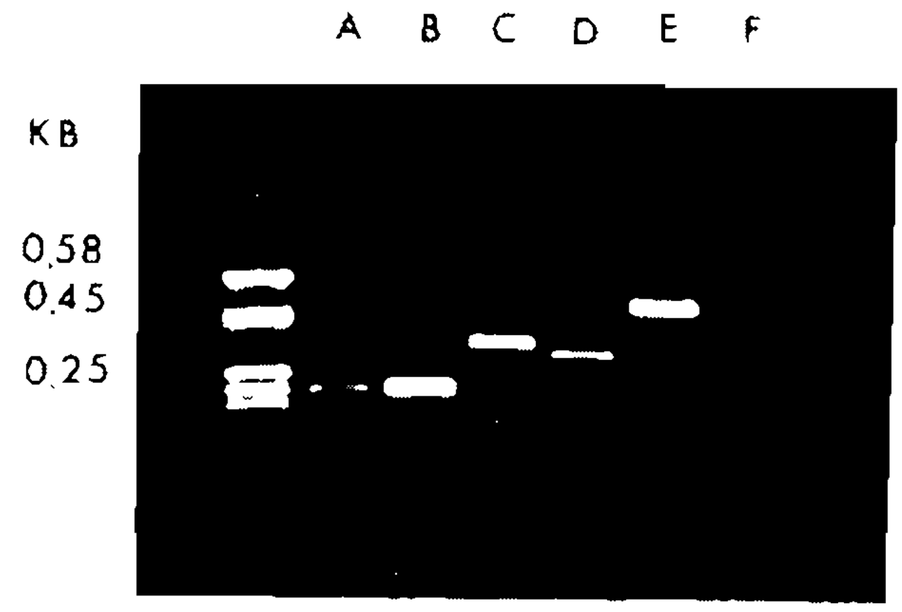

Fig. 4. agarose gel (1.2%) electrophoresis of mini-exon PCR amplification products from New World Leishmanias. Lane A: *Leishmania braziliensis* M2903; lane B: *L. guyanensis* M4147; lane C: *L. amazonensis* M2269; lane D: *L. mexicana* BEL21; lane E: *L. chagasi* pp75, lane F: negative control (no DNA added to the PCR reaction). Molecular size marker (lane M) is pUC 8 digested with HaeIII.

FIELD TESTS

The aforementioned strategies have been developed by the authors over several years, and reflect the current state in the use of PCR and DNA probes for the detection and typing of *Leishmania*. As mentioned before, several other groups have been working with similar or alternative systems, but altogether, thus far only small scale field tests have been performed by the different groups. Although PCR analysis is, theoretically, the promising technique in order to reach maximum sensitivity, preliminary reports from comparative field studies have not always been that optimistic. Sensitivity of 85 to 100 % has been obtained (Smyth et al. 1992). Sensitivity obtained with aspirates from the lesions seems to be consistently lower than with biopsies. However, we should bear in mind that PCR is still a technique in full development, with innovations coming around very frequently, and a whole new approach for sample processing is at the same time being developed. Technical problems, such as false positives due to the extreme sensitivity of PCR, and contaminations occurring in the laboratory, have not eased the evaluation of the technique, especially under field conditions.

CURRENT PCR SCREENING PROCEDURES

The development of PCR screening procedures is governed by several basic rules and observations. Cross-sample contamination and especially contamination with "amplicons" (amplified products after PCR) must be strictly avoided, as these are the major sources of false positives, due to the extreme sensitivity of PCR. The cost of PCR analysis must be kept to a minimum, as this method must compete with other low cost techniques such as microscopic analysis, delayed type hypersensitivity Montenegro test,

and culturing methods. Moreover, we should consider that the majority of the potential users of a new diagnostic test for leishmaniasis suffer chronically from serious budgetary constrictions and that the majority of leishmaniasis patients are living in rural areas in developing countries, thus rendering field conditions often quite extreme. Both sample treatment and further analysis must be extremely simple, and use only low cost materials.

Clinical sample treatment - Clinical samples should be collected with disposable material, or with material that was decontaminated through flaming and autoclaving. Clinical samples such as aspirates or biopsies should be processed, or stored appropriately as soon as possible. Storage can be done in different ways, but freezing at -20°C is preferred for biopsies, aspirates, phlebotomine vectors etc. In circumstances where this is not possible, the material can be stored (1:1) in a 6M guanidinium-HCL, 0.1M EDTA solution at room temperature or at 4°C for several months (Avila et al. 1991).

Samples (1 mm^3 biopsy, or aspirate) which have been stored frozen should be boiled, prior to PCR analysis, for 15 min in 20-40 ml of a 10mM Tris-HCl; 10mM EDTA (TE_{10}) pH 8.5 solution. After boiling, samples should be spun in a micro centrifuge for 15 seconds and 1-2 µl of the supernatant should be used for the PCR reaction.

Samples stored in guanidine-EDTA solution should be boiled for 15 min in order to obtain complete lysis and to liberate the otherwise concatenated minicircles from the network (Britto et al. 1993) followed by extraction with phenol:cloroform, and DNA precipitation with sodium acetate and ethanol. The nucleic acids thus obtained are dissolved in TE_{10} and a fraction is added to the PCR reaction.

PCR amplification - Each PCR system must have carefully balanced reagent concentrations and buffer in order to reach a maximum sensitivity, which lies at best around 10 starting copies of the target sequence in the reaction mixture. Depending also upon the purity and the complexity of the processed sample, the amplification efficiency per cycle may vary. Usually, 25-40 cycles are needed in order to reach a visual signal through gel electrophoresis and staining.

Detection of PCR products - After amplification, PCR products can be visualized by electrophoresis in agarose gels, followed by staining with ethidium bromide and observation under UV light. Spotting of the PCR mixture after amplification with a dot-blot apparatus on filters and subsequent hybridization with a labeled probe, internal to the amplified fragment, usually improves sensitivity with a factor of 10 and is a good confirmatory control for the PCR result.

PROBLEMS IN PCR ANALYSIS

PCR technology for diagnostic purposes struggles with several difficulties.

False negatives - Clinical samples very often contain substances which can partly or completely inhibit the amplification reaction by the DNA polymerase (Jackson et al. 1991). More refined sample treatment methods and perhaps the use of new types of thermostable enzyme will gradually circumvent this problem. The inclusion of appropriate positive controls in the PCR experiment helps to differentiate negative from false negative samples.

False positives - False positives can be generated basically in two ways. Firstly, the PCR reaction must be carefully balanced in order to reach satisfactory sensitivity and specificity. In the reaction mixture, dNTP's, oligonucleotides, concentration of $MgCl_2$, buffer constitution and pH, enzyme and quantity and purity of the target DNA must be optimized and maintained as constant as possible from one assay to another. Disequilibration of the system results in low sensitivity or in non-specific amplification, yielding several unexpected PCR products with no relation to the desired target sequence. Often, some of these spurious products have similar sizes as the expected product. Also, the generation of so-called "primer-dimers" indicates unbalance. Such false positives can be avoided in part by the use of "hot start" techniques, where the annealing of the primers to the template DNA, during the first cycle, happens at the correct annealing temperature, avoiding mishybridizations between the oligonucleotides and the target DNA, and by carefully controlling reaction conditions. False positives due to reaction unbalance can be distinguished from true positives through a hybridization assay using appropriate molecular probes, internal to the desired amplified fragment.

Secondly, false positives can be a consequence of contamination of the sample or the reaction mixture with either primary target DNA or, much worse and frequent, with the so-called "amplicons". These molecules are pre-amplified products that spread into the laboratory environment after analysis of PCR products. Indeed, one single tube containing, for example, 1 µg of a 150 bp fragment, represents about 6.10^{12} molecules (amplicons) or 600 billion times the detection limit of the PCR assay. To avoid this kind of problems, several alternatives can be followed. The most practical ones include the use of (1) separate areas to prepare clinical samples, PCR assays and manipulation of amplified material, (2) dedicated sets of micropipettes for sample treatment, reaction mixture preparations

etc., using plugged tips in order to avoid aerosol contamination of pipettes, (3) all the reagents for the PCR reaction (enzyme, buffer, dNTP's, double distilled water, oligonucleotides, mineral oil, etc.) separated in aliquots before any kind of manipulation and discarded in case of contamination, (4) a "master-mix" for several experiments, containing all reagents except the enzyme, (5) preparation of the PCR reaction mixture in a UV irradiated chamber, (6) negative controls and "no-DNA" controls in each experiment, (7) chemical methods for destruction of amplicons (psoralen, uracil N- glycosylase system etc.).

PROGNOSIS AND NEW DEVELOPMENTS

It is clear that PCR analysis offers, in principle, nearly unlimited possibilities for diagnosis and typing of infectious diseases. However, as is the case with detection of *Leishmania*, much progress has still to be made before the technique can become operational on a routine basis in normal laboratories. Several technological advances are already apparent (Erlich et al. 1991).

Sample processing - New developments have contributed to simplification of sample storage and pre-PCR treatment, using guanidinium solutions, boiling, etc. Special resins are being commercialized by several companies that should be able to bind specifically DNA in complex mixtures such as blood, permitting one step isolation procedure yielding PCR quality DNA. However, further improvements are still needed to make such methods more reproducible, and to reduce the cost of such resins drastically so as to permit more widespread use with large numbers of samples.

Hybridization detection of PCR products - Non-radioactive detection systems, such as chemoluminescent and especially color reaction systems, will greatly ease this application, yielding results within a few hours.

Automation - The PCR procedure can be automated almost completely. We expect that ELISA type assays for detection, and reverse hybridization for typing, will become routine.

In an ELISA test, a detection probe, linked to the wells of a microtiter plate will capture the desired PCR product containing, for example, biotin or digoxigenin label, followed by detection in a color reaction and reading in a microtiter plate reader. Reverse hybridization can also be used to test the labeled PCR product against a series of typing probes, immobilized as dots or lines on nylon or nitrocellulose membranes (Saiki et al. 1989). After development of the color reaction, correct typing can be deduced from the obtained pattern.

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