Identification of Leishmania species by PCR technique in Isfahan , Iran

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Iran is endemic for both zoonotic cutaneous Leishmaniasis (ZCL) caused by Leishmania major and anthroponotic cutaneous leishmaniasis (ACL) caused by L. tropica . The ancient city of Isfahan is suspected to harbour endemic foci for both ZCL and ACL . Control measures require thorough knowledge of Leishmania ecology and epidemiology . The methods for Leishmania species detection andidentification become crucial. In this study the PCR method with species specific primers was used to identify Leishmania species causing CL In these foci. Thirty three samples were taken from parasitologically proven CL patients. The samples were cultured and then harvested promastigotes were washed 3 times and amplified kDNA products were analyzed by electrophoresis in 2% agarose gel . The gel was stained with ethidium bromide . Among 33 isolates investigated 28 isolates were identified as L.major and 5 isolates were identified as L. tropica . The results concluded that PCR is a crucial method for identification of Leishmania spp , and that Isfahan is endemic for both ZCL and ACL , although the majority of cases being ZCL.

This study was supported partially by TDR/WHO and partially by Center for Research and Training in Skin Disease and Leprosy

Key words: Identification, Leishmania, PCR

Introduction:

Leishmaniasis is an important public health problem in tropical and subtropical countries worldwide, involving 400,00 new cases annually, with 12 million people infected and 350 million at risk(1). In Iran, two species of *Leishmania* are causing cutaneous disease . Because mixed infection with more than one *Lei*shmania species or in some regions there are some species which overlap together, precise taxonomic identification of parasites is essential for epidemiological studies. Classic diagnostic techniques include: Microscopic examination, in vitro culture techniques, Montenegro skin test (LST), serological diagnostic techniques, zymodeme analysis and serodeme analysis(2). Molecular techniques, such as polymerase chain reaction (PCR), offer an alternative approach to the demonstration of parasites in clinical samples. Due to their molecular specificity, detection and genetic characterization of *Leishmania* can be accomplished simultaneously. The PCR is a sensitive and rapid technique which can be adapted for use under conditions of limited resources by applying a low cost approach(3). A unique DNA containing structure, in the mithochondrian of the cell. kDNA is composed of two components, maxicircle and minicircle. Maxicircles 20-40 kb in length, are present in 30-50 copies and carry the genes encoding the mithochondrial enzymes. Minicircles are usually 1kb in length, and are present in 10,000-20,000 copies. a unique DNA containing structure, in the mithochondrian of the cell. kDNA is composed of two components, maxicircle and minicircle. Maxicircles 20-40 kb in length, are present in 30-50 copies and carry the genes encoding the mithochondrial enzymes. Minicircles are usually 1kb in length, and are present in 10,000-20,000 copies. A comparision of the sequenses of minicircles from different *Leishmania* species revealed that there is a rigion of approximately 200bp which is conserved among species, while the remaining sequence varies among species and subspecies. This characteristic has allowed their use as diagnostic probes.clonal minicircle fragments have been used as probes to distinguish organisms at species, subspecies and strain level. In this study species-specific primers were used to direct amplification of variable regions of minicircle kDNA from *Leishmania* parasites responsible for cutaneous leishmaniasis in some Isfahanian patients(4).

Material and methods:

The following procedures were done successively: Specimen collection and preparation, culture, Preparation of crude samples for amplification, Polymerase Chain Reaction (PCR). The primers were complementary to the flanks of the conserved regions and directed the amplification variable regions of minicircle kDNA. Their sequences were as follows:Primer 1: 5 TCGCAGAACGCCCCTACC.

Primer 2: 5 AGGGGTTGGTGTAAAATAGGC

Results:The results showed an approximately 700bp product from L.major(MRHO/su/59/pstrain) and an approximately 850bp product from L.tropica(MHOM/SU/58/strain-OD) and a double bounded product from L. major(MRHO/IR/64/Nadim1) one about 700bp and the other about 850bp(fig.1,2).of 33 unknown isolates, which obtained from 33 cultured samples, the pattern of PCR products in 28 cases(84.8%) was like the L. major's(MRHO/IR/64/Nadim1) and in 5 cases(15.2%) was like the L.tropica's(MHOM/SU/58/Strain-OD).

Discussion:
The PCR product of 28 isolates was like the product of *L.major(MRHO/IR/64/Nadim-1)* with 2 bands. This strain was isolated from *Rhombomys opimus* in 1964 by Nadim et al.

According to the result of this pilot study and of another group it seems that this is the dominant species in Isfahan. However, in spite of two other reference strains used in this study, its PCR product has 2 bands. *Leishmania* minicircles are generally uniform in length, being 700-800 bp. However, little is known concerning the organization and degree of heterogeneity of minicircle kDNA within these organisms. In the absence of sequence data for all *Leishmania* minicircles it is unclear why these primers would produce products of varying length. We can consider some explanations: first, it is likely that one of these bands is a non-specific one, but regarding that the condition for 3 reference strains used in this study, was similar in all experiments, this presumption is excluded. Non-specific products can be produced in conditions that there is a large amount of tag polymerase, or the annealing temperature chosen more than the optimal level. Second, it is likely that this strain is an exeptional one and has minicircles of two or varying length, the possible answer of this is genetic recombination, mutation or even hybridization with another species. Finally, the other hypothesis might be that there is two conserved regions within the minicircles of this strain. As it was shown in this study, we used promastigote forms of *Leishmania* parasitesfor identification of species responsible for cutaneous leishmaniasis, but there are many reports of using amastigotes directly for this purpose, for example many studies have been done by using different suitable primers and biopsy samples. All of these group described PCR as a diagnostic effective, and sensitive tool in diagnosis of cutaneous leishmaniasis which can discriminates *Leishmania* spp, too.

Another advantage of using amastigotes for PCR is that its results obtain within a few hours, but culture requires some days. So, PCR is a helpful tool for a precise, confident diagnosis in a short time.

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تعیین هویت گونه های لیشمانیای جدا شده از اصفهان به روش PCR

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یران کشوری آندمیک برای هر دو نوع لیشمانیوززئونوز باعامل لیشمانیا ماژور ولیشمانیوز انسانی با عامل لیشمانیا تروپیکا می باشد. شهر باستانی اصفهان به عنوان کانونی مشکوک برای هر دو نوع بیماری لیشمانیوز محسوب می گردد. اقدامات کنترلی نیازمند دارا بودن اطلاعات کامل از اکولوژی و اپیدمیولوژی لیشمانیا می باشد. روش های مختلفی برای تعیین هویت انگل های لیشمانیا معرفی گردیده اند که یکی از قاطع ترین این متدها روشPCR می باشد که در این مطالعه با استفاده از پرایمرهای اختصاصی مورد استفاده قرار گرفت. ۳۳ ایزوله انگل از افراد آلوده جداسازی شد و به محیط های کشت انتقال یافت. پس از برداشت انگل در فاز ایستا پروماستیگوتهای لیشمانیا سه مرتبه شستشو داده شدند و پس از استخراج DNA کینتوپلاستی با استفاده از پرایمرهای اختصاصی تکثیرDNA با دستگاه ترموسایکلر انجام شد و محصول PCR با استفاده از ژل آکاروز ۲٪ در کنار سویه های استاندارد آنالیز گردید. در بین ۳۳ ایزوله ، ۲۸ ایزوله لیشمانیا ماژور و ۵ ایزوله لیشمانیا تروپیکا تشخیص داده شد.نتایج موید این بود که PCR یک روش قاطع برای تعیین هویت انگل های لیشمانیا می باشد و این که اصفهان برای هر دو نوع لیشمانیوز جلدی شهری و روستایی یک منطقه آندمیک می باشد .

کلمات کلیدی : تعیین هویت ، لیشمانیا، PCR