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Original Article

Whatman Paper (FTA Cards) for Storing and Transferring *Leishmania* DNA for PCR Examination

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Abstract

Background: Diagnosis of cutaneous leishmaniasis (CL) is often made based on clinical manifestation. Correct diagnosis and identification of the parasite are crucial for choosing the effective treatment and for epidemiological studies. On the other hand, determination of *Leishmania* species is necessary for designing appropriate control programs. Diagnosis by PCR is becoming a 'gold standard'. For PCR preparation, storage and shipments of specimens are necessary. In this study, Whatman filter paper (FTA Card) was used to store and transfer samples for *Leishmania* identification using PCR.

Methods: Among the patients who had CL lesion and referred to Parasitology Laboratory of Emam Reza Hospital, Mashhad, Iran, 44 consented cases with positive results in their direct smear were selected. An informed consent form and a questionnaire were completed and three different types of samples (direct smear, NNN culture, and spot on FTA card) were collected. DNA extraction and PCR were carried out on three different samples from each patient.

Results: PCR results using Whatman paper samples revealed a significant difference ($P < 0.0001$) compared to the culture method but no significant difference was seen between PCR results using samples stored on Whatman paper and direct smears.

Conclusion: The use of FTA cards is simple, rapid, and cost-effective, and can be readily employed for large-scale population screening, especially for regions where the specimens are to be transported from distant places to the laboratory.

Keywords: *Leishmaniasis*, PCR, Whatman filter paper

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Introduction

Diagnosis of cutaneous leishmaniasis (CL) is often made based on clinical presentation with an appropriate history of exposure (1). Traditional diagnostic investigations for individual cases include the search of suggestive history and clinical features, the identification of amastigotes by histology or direct smear, and the growth of promastigotes in culture media (2). Leishmanin Skin Test, (LST) is another diagnostic technique but is of less value for anthroponotic CL than the zoonotic form (3). Diagnosis by the PCR is approaching a 'gold standard' status as novel techniques offering a considerable advantage in the collection and transport of specimens and DNA extraction procedures that are more efficient in individual and field-based protocols. Several authors have reported 100% specificity with increasing overall sensitivity of 92% and 98 % (2, 3). Moreover, using PCR technique the identification of *Leishmania* species and subspecies is possible (2). Diagnosis and identification of the *Leishmania* parasite are crucial to initiate an appropriate treatment against different forms of leishmaniasis, for epidemiological and population genetic studies and for development of genetic markers (4).

In this study, Whatman filter paper (FTA Elute Cards, Tokyo, Japan) was used to store and transfer the samples collected for *Leishmania* identification using PCR.

Materials and Methods

This study was carried out in the Department of Parasitology, Emam Reza Hospital, Mashhad University of Medical Sciences, Iran, and the Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences during 2007-2008. The study population was recruited from the patients with suspected CL lesion who referred to the Parasitology laboratory of Emam Reza Hospital. A questionnaire containing demographic, clinical and laboratory

information was completed for each patient who signed an informed consent form was obtained.

A direct smear was prepared from the lesion and examined for the presence of amastigotes. The patients with negative direct smears were excluded from the study. Another skin sample was taken and cultured in liquid phase of NNN (Novy, Mc Neal & Nicole) media. The NNN medium was incubated at 25 ± 1 °C and examined for the growth of promastigotes at 2-day intervals for up to 4 weeks. The liquid phase of NNN medium containing promastigotes was harvested and washed 3 times by sterile phosphate buffer saline (PBS). The pellet was re-suspended in 1,000 µl of PBS and stored at -70 °C until use. The third skin sample was spotted onto FTA Cards No: WB 120210 and air-dried and then stored at room temperature until use.

Sample application from direct smear

The tissue on the stained slides known to contain amastigotes was scraped off with a sterile scalpel and re-suspended into 100 µl of sterile PBS for DNA extraction.

Sample application from Whatman paper

Based on instruction of Whatman paper (WB120210), the disk out of the sample was punched (2 mm) and transferred into PCR tube and 200 µl of FTA purification reagent was added to the tube and incubated at room temperature for 5 min. Washing with the purification reagent was repeated 2 times and the used detergent was discarded. The disk was then washed twice in TE buffer (10 µl Tris-HCl, 0.1 mM EDTA, pH 8.0) for 5 min. The disk allowed drying at 56 °C for 10 min and the disk was ready to be used directly for PCR amplification.

DNA extraction

One hundred μl of promastigotes in PBS stored at $-70\text{ }^{\circ}\text{C}$ or slide scraped suspensions were added to 200 μl of lysis buffer (100 mM Tris; 1% SDS; 10 mM EDTA; 100 mM NaCl) and 20 μl Proteinase K and incubated at $56\text{ }^{\circ}\text{C}$ for 60 min. Three hundred μl phenol-chloroform (50:50 v/v) was added to lysate's micro tube and centrifuged for 5 min at 5000 rpm in a micro centrifuge. The upper layer was added to an equal volume of phenol and centrifuged for 5 min at 5000rpm. The supernatant was added to an equal volume of isopropanol and 1/10 volume sodium acetate. Following incubation at $-20\text{ }^{\circ}\text{C}$ for 10 min, the sample was centrifuged at 12,000 rpm for 15 min. The pellet was washed in 300 μl 70% ethanol and centrifuged at 5,000 rpm for 5 min and then the pellet was resuspended in 20 μl of sterile distilled water (DW) and stored at $-20\text{ }^{\circ}\text{C}$ until use.

PCR amplification

PCR was performed using a pair of 5' TCGCAGAACGCCCTACC 3' and 5' AGGGGTTGGTGTAATAAGG 3' (Cinnagene, Iran) (7).

Reaction was carried out in a volume of 25 μl containing 1 μl DNA (or FTA paper), 1 μl of each primer, 12.5 μl master mix (Biomol, Germany) and 9.5 μl of sterile DW.

The PCR amplifications were performed in a DNA thermocycler (eppendorf mastercycler gradient, Germany) using 31 cycles of $95\text{ }^{\circ}\text{C}$ for 5 min for a single cycle to ensure complete denaturation, $94\text{ }^{\circ}\text{C}$ for 30 seconds,

$60\text{ }^{\circ}\text{C}$ for 45 seconds, $72\text{ }^{\circ}\text{C}$ for 1 min (repeated for 30 cycles) and $72\text{ }^{\circ}\text{C}$ for 5 min, to ensure complete extraction. Every PCR reaction included 2 positive (*L. major* and *L. tropica* species) and a negative control (Fig. 1).

Amplification products were separated on a 1% agarose gel stained with Ethidium Bromide, using a 100 bp DNA ladder as a marker and visualized using a UV transluminator.

SPSS 17 software was used to apply the Cochran and McNemar tests for data analyzing.

Results

A total of 44 patients (23 males, 21 females) ages 3-69 years (Mean 31, SD= 5.6) with CL lesion confirmed by direct smear microscopy were included in this study.

Promastigotes were grown in 32/44 (72.3%) of the cultures (Fig. 2). All FTA Card samples were also positive in the PCR test (Fig. 3). Forty two (95.5%) patients gave positive PCR results when using the direct smear samples (Fig. 4).

PCR results using Whatman paper samples revealed a significant difference ($P<0.0001$) compared to the cultures samples. A significant ($P<0.002$) difference was seen between culture and direct smear, but no significant difference was seen between PCR results using FTA Card samples and direct smears (Table 1).

The sensitivity of the FTA Card samples for PCR was 100% (44/44), for direct smear samples was 95.5% (42/44) and for culture samples was 72.7% (32/44).

Table 1: Comparison of the result of PCR performed by samples obtained from direct smear, culture and FTA Card samples

Source of DNA for PCR	Direct smear		Culture		Whatman paper	
	N	%	N	%	N	%
<i>L. tropica</i>	39	88.6	41	93.2	41	93.2
<i>L. major</i>	3	6.8	3	6.8	3	6.8
Negative	2	4.6	0	0	0	0
Total	44	100	44	100	44	100

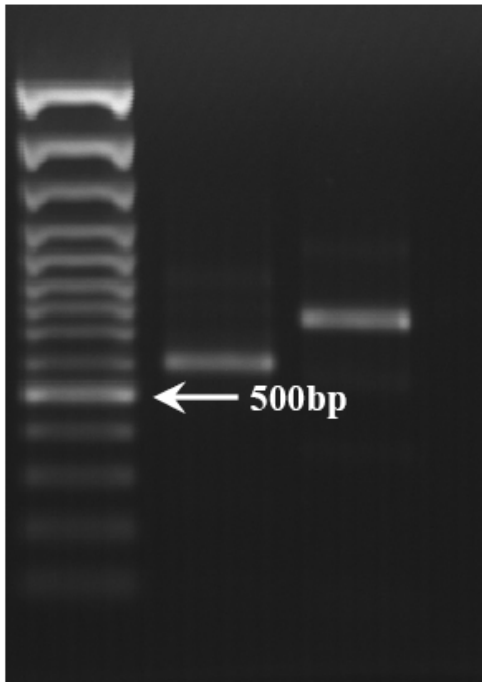


Fig 1: Positive control of *L. major* and *L. tropica* (*L. major*, 600bp and *L. tropica*, 800bp)

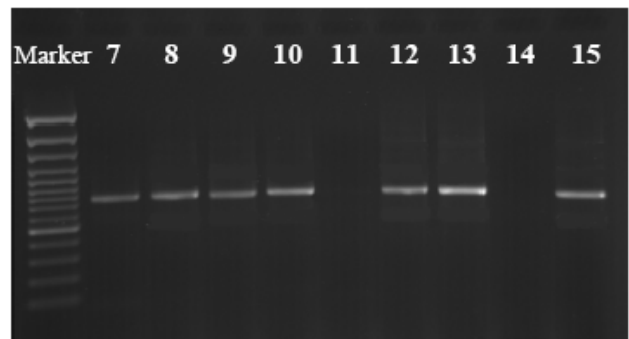


Fig 2: PCR results of Culture. Left to Right: Marker, 7, 8, 9, 10, 11, 12, 13, 14, 15

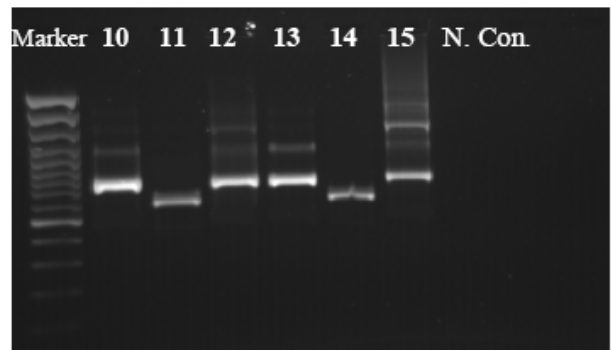


Fig 3: PCR results of Whatman paper. Left to Right: Marker, 10, 11, 12, 13, 14, 15, Negative Control

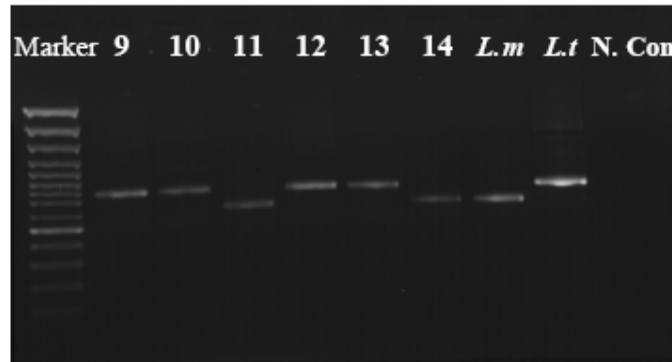


Fig 4: PCR results of Direct smear. Left to Right: Marker, 9, 10, 11, 12, 13, 14, *L. major*, *L. tropica*, Negative Control

Discussion

This study focused on a comparison between different samples of (culture, direct smear and FTA Card samples) used for PCR examination to identify different species of *Leishmania*. Identification of different species of *Leishmania* in an endemic area is necessary for epidemiological control, selecting treatment policy. Standard direct smear is a valuable diagnostic tool for CL, but are not an appropriate tools for sample storage. Concerning the results of previous investigations, it is known that there are at least 3 species of *Leishmania* (*L. major*, *L. tropica* and rarely *L. infantum*) in Mashhad (5-7). PCR using filter paper is shown to be effective in diagnosis of visceral leishmaniasis (8). The disadvantage of culture is the risk of bacterial and fungal contamination, which inhibits promastigote growth (9).

In this study, promastigotes growth was not observed in 12 (27.3%) of cultures, even after 30 days. The result of PCR was also negative for these samples. Similar result was observed which indicated that sensitivity of *Leishmania* culture is not as high as direct smear (10).

Culture needs material, instruments, time consuming and is costly (11). DNA extraction from culture samples also takes time (3-4 h) compared to sample

(3-4 h) compared to sample application from FTA Card (30-40 min).

FTA is a novel tool for collection, shipment; archiving and the stored samples are suitable for purification of nucleic acids.

Comparison of the result of PCR performed by three different samples obtained from direct smear, culture and FTA Card and analysis of the data by Cochran Q test showed that there is a significant difference between the groups of samples ($P < 0.0001$), (Table1). However, by using McNemar test there was no significant difference between PCR results using FTA Card and direct smear samples, but significant difference was observed between direct smear and culture ($P=0.002$) and between culture and FTA Card ($P = 0.0001$). Like culture samples, DNA extraction from direct smear needs more time and sophisticated technique compared to FTA Card's sample.

FTA showed to be effective in sampling, recovery of viral pathogens (12).

In conclusion, obtaining samples using FTA cards is a convenient, fast, safe, and easy method for storage and transportation of the lesion biopsy specimen and DNA extraction. Risk of the contamination is reduced using this method. Large quanti-

ties of DNA could be obtained from the FTA cards and by a faster and easier method compared to the other samples. Samples on FTA cards might be stored at room temperature. The use of FTA cards is simple, rapid, and cost-effective, and might be effectively employed for large-scale population screening, especially for regions where the specimens are to be transported from distant places to the laboratory. Nonetheless, it is not yet known how long the samples can be preserved.

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