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Comparison of three different DNA extraction methods from positive smears prepared from lesions of patients with cutaneous leishmaniasis

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ABSTRACT

Cutaneous leishmaniasis is a dermatic parasitical infection caused by a vector-borne pathogenic patasite.The best and simplest identification method is sampling from lesion borders and taking tissue containing leishmania and prepared smear-positive smesrs are used for DNA extraction. Since DNA extraction is an important stage in molecular diagnostic test, obtaining favorable DNA requires a sensitive and easy as well as cost effective method .The aim of this study was comparing different extraction methods for identisication of different leishmania species. Our results show that although boiling method, despite simplicity and cheapness cannot be used for DNA extraction from smears because of its poor quality. The phenol-chloroform based methods are as valuable as DNeasy mini kit (Kiagene) but more cost effective than kit.The advantages of DNeasy mini kit are simplicity and less time consuming compare with phenol - chloroform based methods.

Key words: Lishmaniasis, DNA Extraction, Phenol:Choroform method, Boiling

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INTRODUCTION

Leishmaniasis is caused by a vector-borne pathogenic patasite found in 88 countries worldwide (1). Cutaneus Leishmaniasis, the most common form of Leishmaniasis, is caused by single celled parasites which are spread by the bite of phebotamine sand flies. It occurs most commonly in Iran, Afghanistan, Syria, Saudi Arabia, Peru and Brazil. Leishmania tropica and Leishmania major are the most common cause of cutaneous Leishmaniasis in Middle East, North Africa and Asia(2).

The parasite Leishmania exists at least in two forms: amastigote form, that is ovoid and non-flagellated form of Leishmania, which is seen in vertebrate macrophages, and flagellated promastigote form, which is seen in the sand fly host and culture media. The transformation of amastigotes to promastigotes starts within hours of ingestion of the amastigotes and occus exclusively in the gut (3). The primary reservoir hosts of Leishmania are sylvatic mammals, such as forest rodents and wild canids (4).

The different species of Leishmania are morphologically indistinguishable but they can be differentiated by DNA sequence analysis(5). Here we analyzed the existence of Leishmania in suspected cutaneoues Leishmania lesions

and employed 3 different DNA extraction methods. then we did polymerase chain reaction for evaluating these methods.

METERIALS AND METHODS

2-1.Smear preparation and Geimsa staining

In this work, sampling was done from patient who were suspected to be infected by leishmania. The aspiratrion was done from edges of the skin sores, where Leishmania parasite is infecting macrophages. The smears ware fixed in methanol for 1 minute and air dried. Geimsa staining was done and microscopy–positive selides was chosen for DNA extraction.

2-2.DNA extraction

DNA extraction was done by three methods: phenol- chloroform, Boiling, and KIAGEN DNA extraction kit.

2-2-1.Phenol: Chlorophorm

This method was a modified procedure of standard phenol:chloroform method [6] as follow: 100µL of lysis buffer was poured on a microscopy positive slides and then the buffer were transmitted to a 1.5 cc tube. 200µL of lysis buffer and 20µL of proteinase K (10mg/ml) was added to the tube. The tube was placed in a bain-marie of 56^{oC} for 1hour. An equivalent valume of phenol:chlorofrom:isoamylalchohol (25:24:1) was added and mixed thoroughly and centrifuged at 14000 g for 1 minute. The supernatant was transferred into a new 1.5cc tube and an equivalent of chloroform:isoamyl alcohol (24:1) was added. Centrifuging was done again at 14000 g for 1 min and aqueous phase was tranferred to a clean 1.5cc tube. An equivalent of 96% ethanol was added and mixed thoroughly and then were placed at -70 ^{oC} for 30 minutes. After 30 minutes, the centrifugation was done at 14000g for 5 minutes and supernatant was discarded. 300µl of 70% ethanol was added to the pellete and again centrifugation was performed at 14000g for 15 minutes. The supernatant was removed and the pellete was air dried. Then 50 µl of distilled water was added to the tube. Then the samples electrophoresed in TBE buffer in a 1% agarose gel.

2-2-2.The boiling method

100µl of lysis buffer was poured on a microscopy positive slides and the buffer were transmitted to a 1.5 cc microtube. the microtube was placed in a bain-marie of 56 for an ouernight. Then the microtube wes placed in 95^{oC} for 10min. After that the sample was centrifuged at 13000g for 2min. Then the samples electrophoresed in TBE buffer in a 1% agarose gel [7].

2-2-3.Extraction by KIA gene DNA extraction Kit

The extraction was done according to manual provided by the manufacturer.

2-3.PCR assay

PCR reactions were done in 25 µl final valume (10x buffer 2.5µl, dNTP 25mM 0.5µl, Primer –F 10µM, 0.5 µl, primer-R 10µM, 0.5µl, taq DNA polymerase 5U/µl 0.5µl, dH_2O 19.5µl, template DNA1 µl).

The cycles condition was as follows: 95^{oC} for 3min for initial denaturation, 30 rounds of : 95^{oC} for 30sec, 60^{oC} for 30sec and 72 ^{oC} for 1min. for denaturation, annealing and extension respectively. Finally the samples were kept in 72 ^{oC} for 5min as final extension. the sequence of primers was as follows:

Forwars primer: 5´ TCG CAG AAC GCC CCT ACC 3´

Reverse primer: 5´ AGG GGT TGG TGT AAA ATA GG 3´

The size of the resulting PCR products by these primers for L.major and L.tropica will be 600bp and 800bp respectively. The PCR products were analyzed on a 1.5% agarose gel along with 1-kb ladder.

RESULTS AND DISCUSSION

3-1-Characterization of Extracted DNA

After DNA isolation, the concentration and purity of DNA were characterized by measuring the absorbance of samples at 260 and 280 nm. The ratio A260/280 of samples purified by boiling, phenol-chloroform and KIAGEN kit was 1.5, 1.7 and 1.8 respectively.

3-2-PCR assay

The exatracted DNA, was then amplified by the mentioned primers. The result can be seen in fig 1.

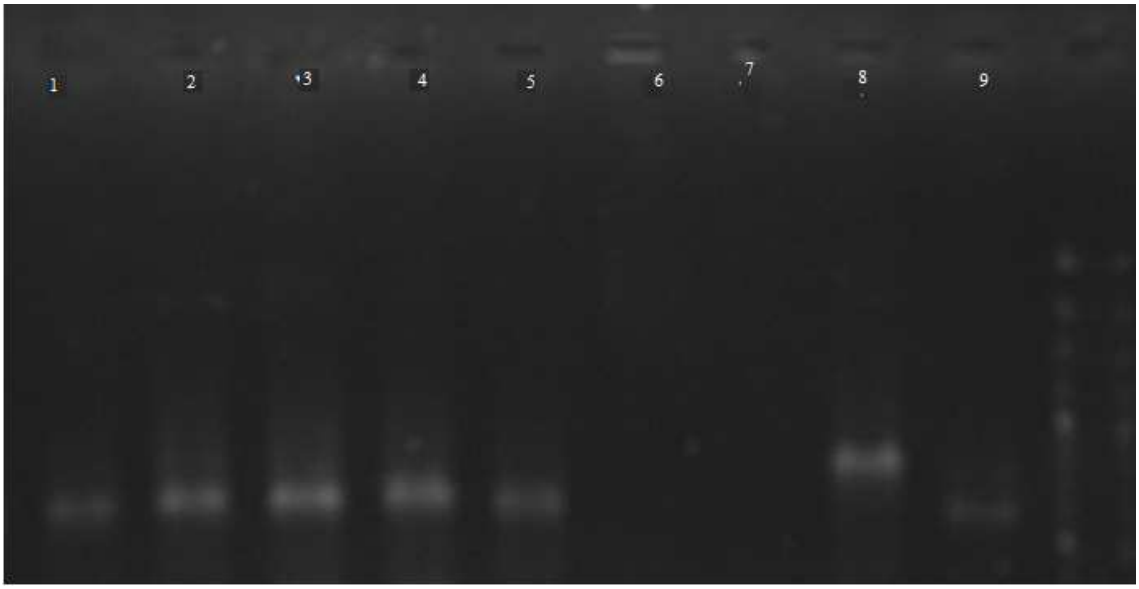

Fig1: PCR products on an 1.5% agaros gel: The first five columns are the PCR products of DNA extracted by phenol chloroform method. Lines 6&7 are the PCR products of DNA extracted by boiling method. Lines 8&9 are the PCR products of DNA extracted by boiling method

CONCLUSION

The Leishmaniasis are considered to be endemic in 88 countries (16 developed and 72 developing countries) on four continents. Today about 12 million cases of Leishmaniasis exist world wide with an estimated number of 1.5-2 million new cases occurring annually[8].

Identification of the parasite species has been a great challenge. Clinical symptoms, the disease epidemiology, vector analyzing, growth in culture media and the ability of causing disease in laboratory animal have been used for this purpose[9]. Molecular detection has revolutionized the diagnosis and identification of infectious agents. By designing proper primers we can easily diffrentiate the species of Leishmania by PCR. The quality of template has a great influnce on PCR results. So, in this research we analyzed 3 different DNA extraction methods. Being easy and cheap, bioling method seems to be a good method for this purpose. But, as our data shows, the quality of DNA obtained from this procedure is very poor in most cases, PCR failed to be done. Phenol-chloroform extraction and KIA GEN DNA extroction kit both yielded a good quality DNA which had good PCR results. So the use of either methods is good but each method has its advantages and disadvantages. Phenol-chloroform method is so effective at extracting the large amounts of DNA. It can be used on a wide range of samples too. However, being very labour intensive, being easily contaminated and exposing the researcher to dangerous chemic has many advantages like reducing time and efforts, but it‘s so expensive. Another disadvantage of kit is that the researcher can‘t change parameters easily.

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