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ORIGINAL ARTICLE

PCR Based Detection of Cutaneous Leishmaniasis

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ABSTRACT

Background: The second most common illness after malaria that is transmitted by vectors and that may be lethal if untreated is leishmaniasis. Leishmaniasis is caused by parasites of the genus Leishmania, which are classified into two subgenera, Viannia and Leishmania.

Methodology: A total of 100 sample collected from presumed clinically as CL patients by dermatologist, with 50 positive cases and 50 negative cases on Geimsa stain microscopy for Leishman Donovan (LD) bodies. After permission from Ethical board in Khyber medical university KPK and inform consent from patient or their guardian in case of children was taken. The lesion and its surroundings were cleaned with 70% ethanol, and the fluid oozing out of the first prick was obtained and disperse over a glass slide, left to dry and fixed with methanol, and stained with Giemsa stain. Two pricks were administered with a sterile BD syringe at the active margin of the lesion. The second prick's fluid was collected and kept in an EDTA tube for PCR usage at -20°C at the IBMS laboratory of KMU in Peshawar.

Results: Out of 100 CL patients, 53 were female and 47 were male. The patients age was ranged from two year to 89 year. PCR was positive in 64 cases and negative in 36 cases out 100 for CL. Out of 50 positive microscopic cases 37cases were positive on PCR. Out of 50 microscopic negative cases 27 (54%) were positive by PCR. A statistically significant difference was found between PCR and microscopic method in the detection of CL.

Conclusion: The finding in our study specifies that PCR base analysis is superior to microscopy for diagnosis of CL. Therefore, we recommend use of PCR for the detection of CL along with Giemsa staining in order to improve the quality and sensitivity of routine diagnosis of the disease.

Keywords: Polymerase Chain Reaction, Giemsa staining, Cutaneous leishmaniasis

INTRODUCTION

The second most common illness after malaria that is transmitted by vectors and that may be lethal if untreated is leishmaniasis (1). Leishmaniasis is caused by parasites of the genus Leishmania, which are classified into two subgenera, Viannia and Leishmania. Female phlebotomine sand flies are responsible for transmitting leishmania (2). There are over 20 distinct species of the Leishmania parasite that are known to exist; however, the ones that are most often found in Pakistan include "Leishmania tropica (L. tropica), Leishmania major (L. major), Leishmania donovani (L. donovani), and Leishmania infantum (L. infantum)" (3). Mammals are the presumed to be the disease's only primary hosts, while reptiles may also get the illness (4). Despite the fact that humans are accidental hosts, there are occasional reports of anthroponotic transmissions occurring without the help of an animal host (5).

There are about 350 million individuals living in locations where they might get leishmaniasis. The number could be higher. Each year, 70,000 fatalities and between 1.5 and 2 million new cases are recorded. In the globe, 2.4 million individuals are affected (6). Almost 90% of all CL instances in the globe are found in Pakistan, Saudi Arabia, Afghanistan, Peru, Iran, Syria, and Brazil, according to research (6). Between 21,000 and 35,000 of the CL cases worldwide are caused by anthroponotic and zoonotic diseases in Pakistan. Punjab, Azad Jammu Kashmir, Khyber Pakhtunkhwa, Federally Administered Tribal Areas, and Baluchistan are the regions with the most instances recorded from Pakistan (7,8). Recurring outbreaks of leishmaniasis are caused by L. tropica in KP and Punjab (9,10). The zoonotic CL infection caused by L. major is prevalent in the south and originates in Sindh, Baluchistan, and Punjab's rural and semi-urban regions (11,12).

Climatic and environmental changes, deforestation, immunosuppression due to HIV, immunotherapy, warfare, natural catastrophes including floods and earthquakes socioeconomic situations, movement to and from countries with prevalent leishmaniasis, and treatment resistance to leishmaniasis are key factors in its spread (13).

For the detection of CL, many laboratory techniques are available. Giemsa staining is the most typical. Nevertheless, it was shown that PCR was more sensitive and may complement other diagnostic methods in the diagnosis of CL. In comparison to other diagnostic techniques, PCR is thought to offer 100% specificity for cutaneous leishmaniasis and sensitivity that has increased by 10% from 20% to 30%. Mucosal leishmaniasis may also be diagnosed with PCR, but unfortunately, PCR is only accessible in advanced laboratories (14.15). For the diagnosis of cutaneous leishmaniasis, a single conventional PCR or its nested variant is often utilised. Various gene sequences have been identified, and PCR can quickly target and find them. Leishmania's kinetoplast (k-DNA) has a variety of known sequences that may be selected for PCR testing (16). Leishmania diagnosis with PCR can identify 0.02 amastigotes per microgram of tissue, demonstrating that it can surpass the limitations of immunological and other traditional diagnostic methods. In labs where limitations and difficulties with traditional diagnosis may arise, PCR may be employed as a consistent and accurate detection tool for leishmaniasis diagnosis (17).

METHODS

A total of 100 sample collected from presumed clinically as CL patients by dermatologist, with 50 positive cases and 50 negative cases on Geimsa stain microscopy for Leishman Donovan (LD) bodies. Most of the patients were from District Nowshera and some from Peshawar KPK Pakistan. After permission from Ethical board in Khyber medical university KPK and inform consent from patient or their guardian in case of children was taken. The lesion and its surroundings were cleaned with 70% ethanol, and the fluid oozing out of the first prick was obtained and disperse over a glass slide, left to dry and fixed with methanol, and stained with Giemsa stain. Two pricks were administered with a sterile BD syringe at the active margin of the lesion. The second prick's fluid was collected and kept in an EDTA tube for PCR usage at -20°C at the IBMS laboratory of KMU in Peshawar.

Direct examination under microscopic: Giemsa stain slides were analyzed under 100X oil immersion objective. All the slides were analyzed twice confirm 50 positive and 50 negative cases of leishmaniasis by observing Leishman Donovan (LD) bodies.

DNA preparation for leishmania PCR: After combining 600µl of cell lysis buffer with 300µl of exudate from a frozen EDTA tube, the mixture was centrifuged at 5000 rpm for 5 minutes. The supernatant was discarded, and the precipitate underwent the aforementioned procedures twice. The clear precipitate was then dissolved in 400 ml of nucleus lysis buffer, 100µl of 5 M NaCl, and 600µl of chilled chloroform. Centrifugation was then performed for one minute at 14000 rpm to separate the upper aqueous layer, which contained about 450µl. This was then combined with 1000 µl of previously chilled 100% ethanol. The tube was put through the procedure described above with 500 µlof 70% ethanol after the ethanol in the tube was discarded. At room temperature, the tubes were dried. These dry tubes were dissolved in 100 µl of TE buffer and now contained precipitated extracted DNA. The extracted DNA was verified and identified using agarose gel electrophoresis and nanodrop electrophoresis. The DNA may be seen using a UV transilluminator.

Polymerase chain reaction (PCR): A conserved region of kinetoplastic minicircle DNA was prepared for PCR amplification for detection of Leishmania tropica, Leishmania infantum and Leishmania major. PCR amplification was carried out with Green Taq Master Mix 10µl, nuclease free water 8µl, LTMI forward primer 0.5µl (5′-GGGTTGGTGTAAAATAGGG-3′), LTMI reverse primer 0.5µl (5′-CAGAACGCCCCTACCC-3′), 1µl of template DNA all added together and this mixture was placed in PCR machine (Thermocycler). This Mixture was incubated for 5 min at 95˚C, followed by 35 cycles, each one consisting of 30 s at 95˚ C, 40 s at 63˚ C, 1min at 72 0 C and final extension for 7 min at 72˚C. Then PCR product held at 4˚ C. Earlier to this PCR, a PCR was carried out for β-globin with above maxing condition of LTMI, except adding specific primers for β-globin forward primer 0.5µl (5′-GAAGAGCCAAGGACAGGTAC -3′), and β-globin reverse primer 0.5µl (5′- CAACTTCATCCACGTTCACC -3′) for 35 cycles (95˚ C for 30 s, 63˚ C for 40 s and 72˚ C for 1 min). Cross-contamination of samples was monitored in each experiment by negative control and positive control. Positive controls comprise of DNA extracted from CL parasites.

Ten microliters of PCR amplified products produced were added by ethidium bromide stained and run on 2% agarose gel electrophoresis. DNA ladder of 100bp was loaded (6 µl) as marker. Gel was visualized under UV light and the amplified fragments of DNA were visualized as bright bands ("750 bp for leishmanial k-DNA while 280 bp β-globin").

RESULTS

Socio-demographic characteristic of Patients: All the samples were 1st subjected to microscopic examination at IBMS, Khyber medical college, KPK, Pakistan. Samples drain from 100 patients 50 positive on slide and 50 negatives on slide (Geimsa stain) for CL were subjected to PCR. And the results of these two different diagnostic procedures Microscopy and PCR were compared. The results obtained with each procedure given in table 1. PCR was positive in 64 cases and negative in 36 cases out 100 for CL. Out of 50 positive microscopic cases 37cases were positive on PCR. Out of 50 microscopic negative cases 27 (54%) were positive by PCR. A statistically significant difference was found between PCR and microscopic method in the detection of CL.

The demographic Characteristics of patients presented in table 2. Out of 100 CL patients, 53 were female and 47 were male. The patients age was ranged from two year to 89 year. With highest frequency of CL was in young ages ranging from 2 to 12 years, which was 51%. No case of CL was reported in age groups 57 to 67 and 68 to 78 in current study. Most of the patients were illiterate (38%), those who had primary education were 52%, and those with secondary school education were only 10 %. Regarding occupation students were more affected 52%.

Individuals who had face lesions were 48 patients followed by 45 patients lesion on upper extremity, 23 lower extremities and 5 patients had lesion on other sites. Total number of patients seem to exceeds our sample size because there were patients who had more than one lesions ether on same site of body or at different sites of body. Regarding number of lesions one, two, three, and more than three lesions at same time were found in 55, 26, 8,10 patients respectively. Only 3 patients had history of lesion from less than a month period, while 97 patients had history of lesion for more than a month.

Out of 100 participants 52 patients had positive family history for leishmaniasis.

Table 1: Comparative diagnosis of leishmaniasis based on microscopic and PCR

	Slide positive	Slide negative	Frequency
PCR positive	37	27	64
PCR negative	13	23	36
Total	50	50	100

Table 2: Sociodemographic Characteristics of Patient (**n**=100).

Characteristics		Frequency (No.)	Percent (%)
Gender	Male	47	47.0
	Female	53	53.0
Age in Year	2-12	51	51.0
	13-23	22	22.0
	24-34	9	9.0
	35-45	10	10.0
	46-56	7	7.0
	57-67	-	-
	68-78	-	-
	79-89	1	1.0
Education	Illiterate	38	38.0
	Primary	52	52.0
	Secondary and above	10	10.0
Occupation	House wife	24	22-0
	Student	50	52.0
	Govt. Services	1	1.0
	Live stock	0	0.0
	Others	25	25.0
Site of Lesion	Face	48	
	Upper extremity	45	
	Lower extremity	23	
	Other sites	5	
Number of Lesion	One	55	55
	Two	26	26
	Three	8	8
	>Three	10	10
First appearance	<month	3	3
	>month	97	97
Family history	negative family cases	48	48
	positive family cases	52	52
Split of positive Family history	One patient	30	30
	Two patients	7	7
	Three patients	6	6
	>Three patients	9	9

PCR was performed on exudate collected from CL lesion. PCR amplification and Gel electrophoresis were considered to be positive for β-globin in those samples which produce a 280-bp band Fig. 1 and consider positive for Cl in those cases, which produced 750bp band Fig 2. These bands were absent in the negative controls. Fig. 1 and 2 shows examples of agarose gel electrophoresis image. All the positive samples were identified strongly as CL based on the bases of homology with reported kDNA sequence.

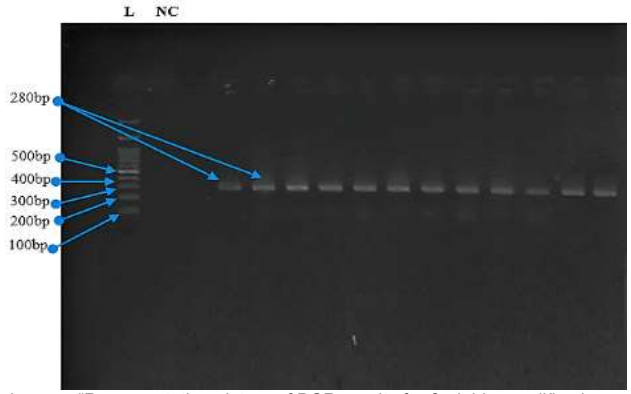

Figure 1: "Representative picture of PCR results for β-globin amplification. In this figure L is 100bp DNA ladder, NC is negative control and the rest of the bands are β-globin 280bp".

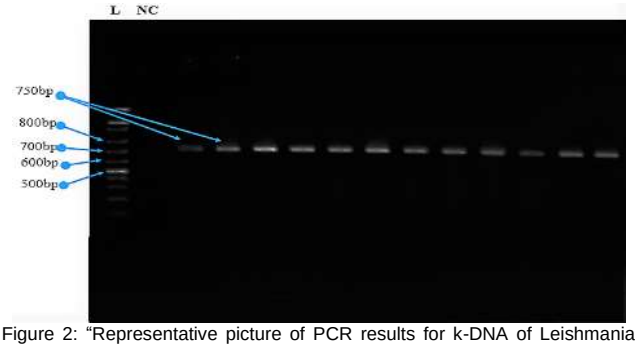

Figure 2: "Representative picture of PCR results for k-DNA of Leishmania amplification. In this figure L is 100bp DNA ladder, NC is negative control and the rest are all positive samples for Leishmanial k-DNA amplicon size 750bp".

DISCUSSION

Because to its excellent sensitivity, microscopic analysis of Giemsa-stained slides continues to be the gold standard method for CL diagnosis. This approach is straightforward and does not need expensively furnished labs. Furthermore, it is quite challenging to identify LD bodies in the majority of CL instances. Incorrect CL parasite diagnosis with this approach might sometimes lead to poor disease treatment. Also, doing microscopy requires a lot of effort and takes a lot of time. In circumstances when the number of parasites is minimal, PCR is a more sensitive diagnostic method than microscopy. While CL microscopy findings are negative, PCR may nevertheless amplify CL DNA from extremely low parasite numbers. Hence, PCR might be useful in low parasite infection patients that the physicians thought to be CL but that could not be determined by microscopy.

For appropriate treatment options and the development of effective treatments, accurate identification of Leishmania species is necessary (33). Early CL identification and accurate diagnosis for successful treatment are crucial parts of CL management and are necessary to break the cycle given the rising prevalence of medication resistance in endemic settings. By using PCR methods, the CL parasites may be found with an almost perfect specificity (34).

The overall cost of PCR in Pakistan is not as high as compare to develop countries. A basic equipped molecular biological lab with minimum infrastructure including a thermocycler, a gel electrophoresis apparatus and a UV transilluminator are one-time expenditure. If PCR equipment's available in laboratories of endemic areas, it could be performed as routine diagnostic procedure.

Early investigations agreed that PCR coupled with the conventional Giemsa staining test demonstrated better sensitivity of 74% with better outcomes in comparison to direct microscopy only(18.19,20,6). In contrast to some studies a study from Sindh show more female patients than male, our study and some of the other studies had similar finding (2122,23,24). This may be due to demographic variables and the exposure behaviors that both sexes are likely to adopt. Face was the prominent area of bite as higher frequency noted with 48%, followed by 45% in upper extremities and 23% of lesions on lower extremities. This type of finding seen in other repots also (24,25). Due to outside activities and higher probability of exposure to the environmental factors young age groups are at higher risk of CL as reported commonly (26,27). We found highest frequency of the infection 51% in the range of 2-12 years followed by 22% in the age group 13-23 in ours study.

Most of the CL patients noted to be illiterate (38%) or had primary education (52%), with higher education level of secondary school and above were only 10 %, as reported similar findings in many studies, they reported high incidence in less educated people (28,29). Because these people are not aware of preventive measure or they are mostly young age, preschool children are biasing data by adding bulk to illiterate category. We identified the various jobs of leishmaniasis patients, and it was discovered that students had the illness 50 out of every 100 times, followed by other occupations like farmers, wood cutters, and labourers, etc., at 25 out of every 100 instances, housewives at 24, and government employees at 1. Just one out of every 100 cases of the disease was found in the group of government employees. Again the high number of students is due to young age more at risk, this is what mostly reported (30,29). Most of the patients in endemic area were multiple time exposed to bite as in 44 % of cases had more than one lesion on their body at the time they attended medical help, with breakup of two lesions in 26 cases (26%), three lesions in 8 cases (8%) and more than three in 10 cases (10%), Kumar et al., 2018 had similar report (25).

There was strong connection of spread of CL in closed environments as we found 52 patients with positive family history of CL patients. This suggests that risk variables had a significant role in the infection rate in previously infected households, and that a plan should be developed to identify and reduce these risk factors. These results from our findings were much higher as compare to other studies, this may be due to their relatively large sample size or other environmental factors and knowledge, attitude and practices of CL patients in those environments (22,31).

CONCLUSION

PCR is more reliable option for diagnosis and this procedure should be decentralize to the district level or specifically to endemic areas, so that we can be confident in CL detection and treatment. It is very essential to develop a less invasive or non-invasive methods as source for PCR in endemic countries. The finding in our study specifies that PCR base analysis is superior to microscopy for diagnosis of CL. Therefore, we recommend use of PCR for the detection of CL along with Giemsa staining in order to improve the quality and sensitivity of routine diagnosis of the disease.

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