Diagnostic Accuracy of Polymerase Chain Reaction Against Giemsa Staining on Tissue Biopsy for Cutaneous Leishmaniasis

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ABSTRACT

Objective: To evaluate the diagnostic accuracy of a commercial real-time polymerase chain reaction (PCR) kit targeting 18S rRNA against Giemsa-stained tissue slides in patients clinically suspected of cutaneous leishmaniasis (CL).

Study Design: Cross-sectional analytical study.

Place and Duration of the Study: Department of Microbiology, Armed Forces Institute of Pathology / National University of Medical Sciences, Rawalpindi, Pakistan, from July to December 2022.

Methodology: Samples of skin tissue in 98 patients suspected of CL were evaluated. These samples were subjected to Giemsa-staining for microscopy and real-time PCR. Sensitivity, specificity, and accuracy of the PCR were calculated keeping Giemsa-stained tissue slide microscopy as gold standard.

Results: Out of the 98 tissue samples, 37 were found positive for leishmaniasis on PCR while 13 were found *Leishmania* positive on microscopy of Giemsa-stained slides. The sensitivity, specificity, and accuracy of the PCR for the detection of *Leishmania* species were 100%, 71.8%, and 91.8%, respectively with 100% negative predictive value.

Conclusion: This study demonstrates that the commercial PCR is a reliable diagnostic test for the diagnosis of CL. The ease, rapidity, and reliability of the PCR make it a dependable tool in diagnostic repertoire of CL.

Key Words: *Giemsa stain, Leishmania spp., Polymerase chain reaction, Viasure.*

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INTRODUCTION

Leishmaniasis is the third most significant global vector-borne disease which spreads *via* infected sandfly bites.[1,2] There are over 20 *Leishmania* species causing human infections.[1] This infection, found in approximately 88 countries, predominantly thrives in regions characterised by tropical and subtropical climates. Among these regions, Afghanistan, Brazil, Iran, Saudi Arabia, Peru, and Syria stand out with over 90% of reported infections originating from these nations.[3] It manifests in various forms including visceral (VL), cutaneous (CL) and mucosal (ML). Cutaneous leishmaniasis (CL) is the most prevalent among them, accounting for approximately 0.6 to 1 million new cases reported annually.[4]

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CL manifests as a skin disorder characterised by the appearance of one or more lesions, typically presenting as ulcerated or verrucous plaques, on parts of the body within the reach of the vector sandflies. In some cases, the infection may extend to involve the lymph nodes and satellite lesions may also develop, *albeit* infrequently.[5]

Cutaneous form of leishmaniasis is more widespread in Pakistan.[6] In the 2013-2015 epidemic, war-affected Waziristan saw a 3.61% prevalence of CL.[7] A cross-sectional study conducted across four Khyber Pakhtunkhwa districts including Karak, Lakki Marwat, Tank, and Dera Ismail Khan revealed a 50.4% prevalence of CL among participants with skin lesions.[8] Over the last decade, the country has experienced an anticipated annual incidence of 4.6 cases per 1,000 individuals.[9]

CL is also known as the great imitator, as it can mimic various dermatoses. Such similarity occasionally leads to misdiagnosis, resulting in inappropriate treatment and health issues.[10] This demands early and accurate diagnosis to replace costly and complex treatment regimens with substantial adverse effects and increasing resistance concerns.[11] The gold standard for the diagnosis of CL is the parasite-based tests including microscopy, parasite isolation by *in vitro* culture or inoculation of mice and hamsters.[12] The immunological tests including skin-prick tests

and ELISA for antibody detection are also available with limited reliability.

Currently, molecular techniques for diagnosis of leishmaniasis are being increasingly adopted because of their rapidity.[11,13] Among these methods, the real-time PCR gives ease of usage, rapidity of result, real-time result analysis, and low-risk of contamination. A commercial real-time PCR kit targeting 18S rRNA claims to be highly sensitive and specific for the diagnosis of leishmaniasis. However, it has never been evaluated in Pakistan for its diagnostic sensitivity claims.[4] Therefore, this study was planned to evaluate the diagnostic accuracy of this commercial real-time PCR Giemsa-stained tissue slide microscopy in patients clinically suspected of CL.

METHODOLOGY

This was a cross-sectional analytical study, conducted at the Department of Microbiology, Armed Forces Institute of Pathology / National University of Medical Sciences, Rawalpindi, Pakistan. The sample size was calculated by using Buderer's formula.[14] Considering sensitivity of real-time PCR to be 93.9%, specificity as 100%, prevalence of *Leishmania* spp. as 50.4%, confidence level 95%, and margin of error 5%. The minimum sample size for this study was calculated to be 98.[8]

The sampling method employed in this study was non-probability convenience sampling, wherein individuals were conveniently selected based on their availability and suitability for the research. Patients of all ages and both genders, presenting with documented non-healing ulcers persisting for one month or more, and seeking medical attention at a tertiary care hospital in Rawalpindi, were eligible for inclusion. However, patients who had received partial or complete treatment for CL were excluded from the study population. Prior to participation, individuals provided informed consent and completed a detailed data collection form. Dermatologist then meticulously collected skin tissue biopsy specimens from the margins of the ulcers in a sterile manner which were subsequently dispatched to the study place for further analysis and examination.

These tissue fragments were finely ground using a scalpel and petri dish. Each sample was then divided into two parts. The initial step involved subjecting the first tissue fragment to Giemsa staining for microscopy. Films were made from tissue fragments, dried in the air then fixed by immersing in methanol for 10 minutes. Then the fixed films were transferred to a staining jar containing May-Grunwald stain freshly diluted with an equal volume of buffered water. After the films were allowed to stain for about 15 minutes, they were transferred without washing to a jar containing Giemsa stain freshly diluted with 9 volumes of buffered water. After staining for fifteen minutes, they were transferred to a jar containing buffered water and rapidly washed in four changes of water and left to stand for five minutes.[15] After air drying, the stained slides were examined using a light microscope, employing oil immersion, with the aim of identifying any presence of *Leishmania* amastigotes within the tissue samples. The presence of *Leishmania* amastigotes can be seen in positive samples as shown in Figure 1.

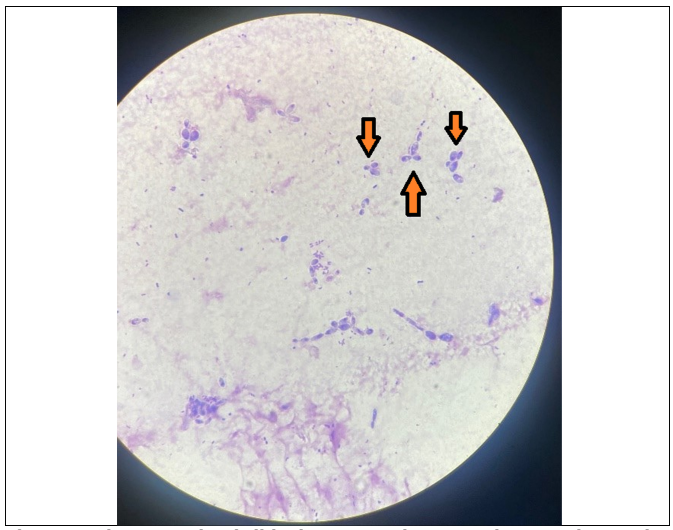

Figure 1: Giemsa-stained slide demonstrating amastigotes taken under X1000 oil immersion lens.

The subsequent segment of the ground tissue specimen underwent real-time PCR analysis. To initiate DNA extraction, the Merck Millipore's Bacterial Xpress Nucleic Acid Extraction Kit was employed.[16] To extract DNA, approximately 200 µl of extraction reagent, along with 50 µl of the sample, and an additional 10 µl of internal standard were combined within a microcentrifuge tube and thoroughly mixed *via* vortexing. Following a five-minute incubation period at room temperature, 250 µl of isopropyl alcohol was introduced into each tube, vigorously vortexed, and subsequently subjected to centrifugation at 16,000g for 10 minutes. Upon completion of centrifugation, the supernatant was carefully discarded and the resulting pellet was subjected to a washing step with 400 µl of 70% ethanol, followed by another round of vortexing and centrifugation at 6,000g for 10 minutes. After the removal of the supernatant, the pellet was air-dried and subsequently re-suspended in 50 µl of water. For the subsequent PCR analysis, a *Leishmania* real-time PCR Detection kit was employed.[17] In each Smart Cycler tube, 10 µl of the DNA extract was combined with 20 µl of the master mix. These prepared tubes were then positioned within the Bio-Rad CFX96™ System RT PCR machine and the amplification process was initiated following the protocol outlined in the kit instructions. It is crucial to note that any sample exhibiting a cycle threshold (Ct) value of less than 40 was deemed positive for the presence of *Leishmania* spp.[4] The positive and negative results were recorded for each sample. Figure 2 shows a positive test.

The data analysis was conducted utilising the Statistical Package for the Social Sciences (SPSS) version 23. For qualitative variables, frequencies and percentages were computed, while for quantitative variables, the mean and standard deviation were calculated. Inferential statistics were employed to assess the diagnostic accuracy of PCR, utilising Giemsa-stained tissue slide microscopy as the gold standard. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy measures were determined to evaluate the performance of the PCR assay.[12] Cohen Kappa was calculated to estimate the level of agreement between the two tests.

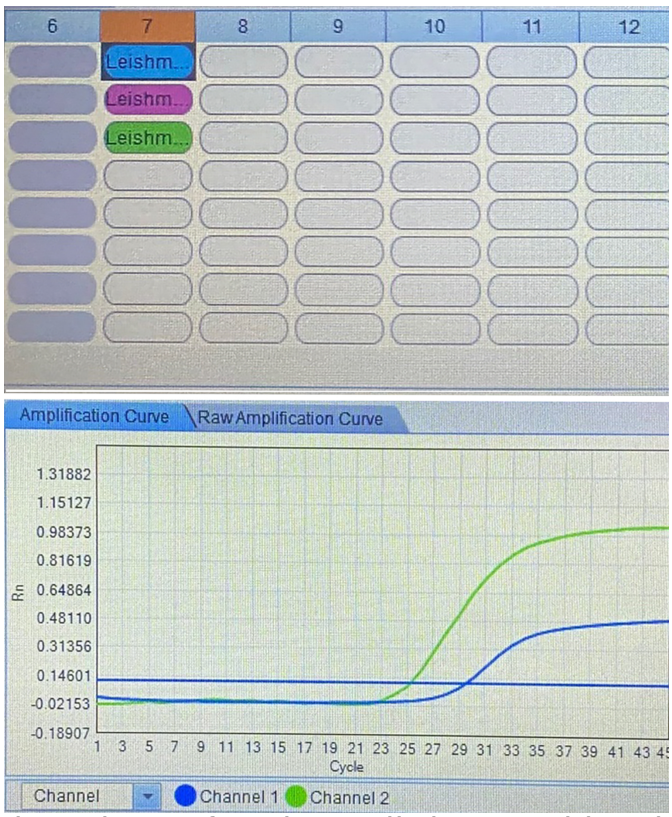

Figure 2: The curves of PCR as interpreted by the system made in a positive sample. Green curve indicates the sample that came out to be positive. Blue curve indicates positive internal control.

RESULTS

Out of the total 98 subjects, the majority were males, constituting 95.9% (n = 94), while female patients accounted for 4.1% (n = 4), with an overall average age of 37.51 ± 12 years. Majority of patients belonged to Rawalpindi (66.30%) followed by Waziristan (23.50%).

A comparison between PCR results and Giemsa-stained slides revealed that out of the ninety-eight samples assessed, thirty-seven samples tested positive *via* PCR, whereas only thirteen samples were positive through Giemsa staining. The sensitivity of the commercial PCR kit for *Leishmania spp.* detection was calculated as 100%, with a specificity of 71.8%. The PPV was determined to be 54.7%, while the NPV was 100%. The overall diagnostic accuracy of the PCR was found to be 91.8%.

Considering that the positivity of CL *via* Giemsa-stained slide microscopy was lower compared to the index test (PCR), Staquet's correction was applied to ascertain the sensitivity and specificity of the PCR under conditions where the reference standard was imperfect.[18]

The sensitivity of the Giemsa-stained specimen was taken to be 37% and specificity to be 100%.[19] Therefore, post-application of Staquet's correction, the ultimate sensitivity of the PCR kit in this study was determined to be 100%, while its specificity was calculated at 97%. Additionally, the Cohen Kappa value, assessing the agreement between the PCR and Giemsa-stained slides, was found to be 0.403, with a significant p-value of <0.001.

DISCUSSION

CL stands as the most overlooked tropical ailment worldwide as it is undiagnosed or is misdiagnosed.[10] The present approaches to treating leishmaniasis lack considerable efficacy and frequently lead to notable adverse effects.[11] Leishmaniasis causes significant morbidity and mortality in low and middle-income countries (LIMCs).[5] Swift and accurate diagnosis, along with the identification of specific species, plays a vital role in halting the advancement of leishmaniasis.[13] The literature indicates that Giemsa-stained specimen microscopy demonstrates high specificity (100%) but its sensitivity is considerably low (37%).[19] This study also indicated the same through the Cohen's Kappa which suggests that both PCR and Giemsa-stained tissue specimens detect CL in a fairly consistent way but there is still room for improvement in the gold standard.

The tested PCR is designed for diagnosis of the genus *Leishmania* targeting 18s rRNA.[4] It identifies the presence of *Leishmania* species but does not differentiate between the specific types. The findings indicated that the tested PCR had a sensitivity of 100%, specificity of 97%, PPV of 54.5%, NPV of 100%, and diagnostic accuracy of 91.8%. In literature, a study was conducted by Arnau *et al.*, in which they evaluated the diagnostic accuracy of the presently studied PCR against *Leishmania* culture and another commercial PCR kit.[4] The specificity in this study is 100%, which is comparable to this study (97%) but sensitivity was found to be much lower (81.8%) as compared to 100%. Likewise, the PPV and NPV were 100% and 72.7% which are quite different from this study.[4] The reason for this difference in results is most likely because of the different reference methods in the two studies.

The gender distribution in this study was approximately 96% males and 4% females which is in contrast to another study conducted in the North Waziristan Agency.[20] In this study, they elaborated the pattern of CL in the North Waziristan agency and found that among microscopically confirmed CL patients, 57% were males while 43% were females.[20] The reason for the difference in gender distribution in both studies is likely because of the difference of patient catchment area.

Comparing the Giemsa-staining results of this study with those of a study by Nateghi *et al.*, where the authors assessed two types of PCR alongside Giemsa-stained specimens, it becomes apparent that their Giemsa positivity rate was 77.27% (17/22), while this study indicates it to be 35% (13/37).[21] This shows a significant disparity in the positivity rates between the two studies. The observed difference in positivity rates could be attributed to several factors, including unequal distributions of parasites within the lesion. It is plausible that the portion of the lesion collected for smear preparation may not have contained enough parasites for detection by microscopy, contributing to the lower positivity rate. Additionally, variation in the expertise of the microscopists involved in the examination of the stained slides could have influenced the detection and interpretation of parasites. Overall, these findings underscore the importance of considering various factors, such as lesion characteristics and

expertise levels, when interpreting Giemsa staining results for the diagnosis of CL.

Among the patients included in the study, 80.6% (79/98) presented with solitary lesions, with or without crusting or purulent discharge, while 19.4% (19/98) had multiple lesions. Interestingly, this distribution contrasts with findings from a study conducted in Iraq by Ali *et al*., where multiple ulcers were more prevalent on the body (60.44%) compared to solitary ulcers (39.66%).[22] One potential explanation for this disparity could be related to the demographic characteristics of the study's patient population. Military recruits were primarily selected, and it is plausible that their health is monitored regularly, allowing for prompt observation and treatment of initial lesions before the development of additional ones. This proactive approach to healthcare may have led to a higher proportion of solitary lesions observed as compared to the Iraqi study.

Despite the accuracy of the tested PCR for diagnosis of CL, the high cost is still a challenge in resource-limited settings, however, the ease of use, rapid turnaround time, and consistent reliability make it a valuable addition to the diagnostic arsenal for this condition, facilitating timely intervention and treatment strategies. For implementation in such resource-limited settings, Giemsa-staining should be the first-line diagnostic tool, however, for patients with chronic skin lesions in areas of high endemicity, PCR can be used as a second-line confirmatory test for definitive diagnosis.

This study highlights that molecular detection using the tested PCR can be used for accurate and timely diagnosis of CL thus preventing the spread of the disease within the country. Based upon the increasing incidence of the disease and the associated morbidity, the association of the clinical picture with the species can be the future avenues of research. Further studies involving species identification and their association with disease activity and treatment response are recommended.

CONCLUSION

This study provides compelling evidence that PCR targeting 18S rRNA is a dependable diagnostic tool for the accurate diagnosis of CL.

ETHICAL APPROVAL:

Ethical approval was taken by Institutional Review Board and Ethical Committee (Letter Number: FC-MIC21-2/READ-IRB/22/1914, Dated: 27 Jun 2022).

PATIENTS' CONSENT:

Consent was obtained from all patients prior to their participation in the study.

COMPETING INTEREST:

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

AK: Conception, interpretation, and drafting of the manuscript.
MMG, IAM: Analysis and revision.
SHN, RS: Interpretation and revision.
SAHG: Conception and drafting of the manuscript.
All authors approved the final version of the manuscript to be published.

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