

Comparative Evaluation of Multiplex PCR, RLEP PCR and LAMP PCR in Urine, Stool and Blood Samples for the Diagnosis of Pediatric Leprosy – A Cross-Sectional Study

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ABSTRACT

Objective: To compare the diagnostic efficacy of Multiplex polymerase chain reaction (PCR), *Mycobacterium leprae*-specific repetitive element (RLEP) PCR and loop-mediated isothermal amplification (LAMP) PCR in the diagnosis of pediatric leprosy as an alternative to slit-skin smear (SSS) examination.

Methods: A cross-sectional study was performed on 26 children aged 0-18 years with characteristic skin lesions of leprosy. SSS examination for acid fast bacilli (AFB) was performed for all children. Additionally, urine, stool and blood samples were tested by three PCR techniques - Multiplex, RLEP and LAMP. The results of these tests were compared with each other and with results of SSS examination for acid fast bacilli (AFB) using appropriate statistical tests.

Results: Out of 26 patients studied, SSS examination was positive for AFB in 7 cases (26.9%). In blood samples, the positivity of Multiplex PCR, RLEP PCR and LAMP PCR was 84.6%, 80.8%, and 80.8%, respectively. Multiplex PCR in blood samples was positive in 100% ($n = 7$) of SSS positive cases and 84.2% (16 out of 19) of the SSS negative cases ($P < 0.001$). The positivity of all PCR methods in urine and stool samples was significantly lesser than in blood.

Conclusion: Multiplex PCR in blood sample is a superior diagnostic tool for pediatric leprosy compared to RLEP PCR and LAMP PCR, as well as SSS examination.

Keywords: *Multibacillary, Mycobacterium leprae, Paucibacillary, Slit-skin smear*

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INTRODUCTION

Leprosy is a chronic disease of moderate infectivity with a long incubation period. *Mycobacterium leprae* has predilection for peripheral nerves and skin [1,2]. Children are believed to be the most vulnerable group due to their nascent immunity and exposure to intrafamilial contacts [3]. The two traditional diagnostic tests for leprosy viz. slit-skin smears (SSS) examination for acid fast bacilli (AFB) and histopathological examination of biopsies, have their inherent limitations. SSS examination is relatively low in sensitivity and carries a risk of subjective errors of microscopic examination, whereas the histopathological examination has the limitations of a long turnaround time

and is technically demanding. Hence, there is a need to develop highly sensitive laboratory tests for early detection of leprosy which will help to prevent transmission and limit deformities [4]. With this background, this study was undertaken to investigate the diagnostic potential of three different molecular techniques, viz Multiplex polymerase chain reaction (PCR), *Mycobacterium leprae*-specific repetitive element (RLEP) PCR and loop-mediated isothermal amplification (LAMP) PCR in urine, stool and blood samples in the diagnosis of pediatric leprosy as an alternative to slit-skin smear (SSS) examination for acid fast bacilli (AFB).

METHODS

This cross-sectional study was performed between September 2020 and March 2022 in the Department of Pediatrics, Sarojini Naidu Medical College, Agra and ICMR-National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra. A total of 26 patients of age group 0-18 years, in whom leprosy was clinically

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suspected as per WHO Guidelines [5] were serially enrolled. A written informed consent was taken from the guardians. Children previously treated with multi-drug therapy (MDT) were excluded from the study. Prior approval was obtained from the institutional ethics committee.

All enrolled patients were subjected to a detailed history and examination including evaluation of skin and any other site of involvement, description of skin lesions and nerve involvement if any. SSS examination was performed for each patient; smears were subjected to Ziehl-Neelson staining for AFB and microscopy. Urine, stool and blood samples of the patients were collected and subjected to Multiplex PCR, RLEP PCR and LAMP PCR. The method of collection of urine, stool and blood samples was adopted as per the Centres for Disease Control (CDC) guidelines [6]. Freshly passed stool specimen (3-5 g) was collected in falcon tubes. Midstream urine sample (15-20 mL) was collected in sterile container. Stool and urine samples were stored at -20°C until testing was performed. Venous blood (2 mL) was collected in EDTA vial and stored at 2- 8°C.

Slit-skin smear examination for AFB, RLEP PCR, multiplex PCR and LAMP PCR of urine, stool and blood sample was performed at the National JALMA Institute of Leprosy & Other Mycobacterial Diseases (ICMR Centre), Agra, India.

Slit-skin smear examination was performed based on the methodology proposed by the International Federation of Anti-Leprosy Associations (ILEP) [7]. Skin smear was taken in a good light, so that the lesions are easily seen, and their color and activity evaluated. The skin area chosen for the smear was cleaned by rubbing briefly but vigorously with a cotton wool swab dipped in spirit. The area was then allowed to dry. A fold of skin (or the bottom of the ear lobe) was picked up between the index finger and thumb and squeezed tightly to prevent blood flow. A cut was made with a scalpel blade 5mm long and 3 mm deep to get into the dermis. The blade of the scalpel was then rotated through a 90-degree angle, and the edges of the slit skin were scrapped several times to obtain tissue pulp. A part of the material was transferred from the point of scalpel onto a clean new glass slide and spread evenly making a circle of 8 mm diameter. The smears were air-dried and then fixed by passing it over the flame of a spirit lamp.

The forward (F) primer used for RLEP PCR was 5'TGC ATG TCA TGG CCT TGA GG 3' while the reverse (R) primer used was 5' CAC CGA TAC CAG CGG CAG AA 3'. For Multiplex PCR primers used were: ML 1545 F (5'GTCTCCGTCTTGCTGACTG3'), ML 1545 R (5'CATACCGGCCATATTGCGTC 3'), ML 2180 F

(5'ACGCTCGCGTATGTCAAAAA 3'), ML 2180 R (5'CGATCACGATGCCGAACCTT 3'), ML 2179 F (5'ATGAACTGCTAGATGTCCGGG 3'), ML 2179 R(5'ATCTTTGCGCGAGTCTTGTG 3'). Each sample was dipped in 400 µL of TE buffer and samples were taken in eppendroff tube. All the purified samples were processed for the isolation of exosomal DNA using van Embden method with modifications [8]. The primers were synthesized by GCC Biotech. Complete amplification of RLEP and multiplex was performed in a 25 µL total volume using specific reaction amplification reaction was done in programmable thermal cyclers with 45 cycles (PTC 150 Minicycler; MJ Research). After the completion of PCR reaction, amplicons were enlarged by electrophoresis and the DNA bands were visualized under UV (ultraviolet) light.

For LAMP PCR, three sets of primers viz. forward and backward outer primers (F3, B3), inner primers (FIP, BIP) and loop primers (LF, LB) were used, viz F3 (5'-GTCAAAAATCGTGCGGTTCC-3'), B3 (5'-CGAAA GCAGGCAGTCAGC-', FIP (5'AACAGCCATTTCC CCACCACCGGGCTCTGTGTCTTGTG3'), BIP (5'TATGTTTCGGTAGTCGTGGGGGCCAAAACC CCGCAACACAG3'), LF(5'ACAATCACCGCC ACAGA-3') and LB (5'-CAGCCCAGGAATCCTGTT GA-3'). For LAMP PCR after sample preparation, lysis and extraction of DNA and amplification were done using the above sets of primers. Postamplification results were visualised by adding 0.1% SYBR Green to the tubes.

Statistical Analysis: Data collected was tabulated using Microsoft excel and analysed using SPSS Version 16.0. Descriptive analysis was used for the analysis. Cohen's kappa was used as a test of agreement to compare the result of slit skin smear examination, RLEP PCR, Multiplex PCR and LAMP PCR on urine, stool and blood samples. Cohen's Kappa result were interpreted as follows: values 0-0.20 as no agreement and 0.21-0.39 as minimal, 0.40-0.59 as weak, 0.60-0.79 as moderate, 0.80-0.90 as strong, and > 0.90 as almost perfect agreement. *Chi-square* test was used as a test of association.

RESULTS

Out of 26 patients enrolled in our study, 20 (76.9%) were aged between 11-18 years of age. The mean (SD) age of the patients was 14.9 (3.4) years, ranging from 8 to 18 years. There were 19 boys and 7 girls. As per Ridley Jopling classification [2], there were 2 (7.7%), 4 (15.4%), 14 (53.8%), 3 (11.5%) and 3 (11.5%) cases of Tuberculoid (TT), Borderline Tuberculoid (BT), mid-Borderline (BB), Borderline Lepromatous (BL) and Lepromatous Leprosy (LL). There were 20 (76.9%) cases of multibacillary and 6 (23.1%) cases of Paucibacillary leprosy as per the WHO

classification of leprosy. A history of family contact was present in 19 (73.1%) of leprosy patients. 10.5% (1 out of 6) of PB cases and 89.5% (18 out of 20) of MB cases had a positive history of contact ($P = 0.012$).

Out of 26 patients, SSS for AFB was positive in only 7 (26.9%) patients. The SSS for AFB was positive in 7 out of 20 (35%) of MB cases and was negative in all PB cases ($P < 0.001$).

In blood samples, the positivity of Multiplex PCR, RLEP PCR and LAMP PCR was 84.6%, 80.8%, and 80.8%, respectively. The corresponding figures in urine samples were 34.6%, 26.9%, and 30.8% and in stool samples were 15.4%, 7.7%, and 11.5% (**Table I**). Multiplex PCR in blood was positive in 5 out of 6 (83.3%) of PB cases and 18 out of 20 (90%) of MB cases ($P = 0.654$). The corresponding figures in urine were 16.7% and 40% ($P = 0.292$) and in stool samples were 16.7% and 15% ($P = 0.921$).

Table II depicts the diagnostic yield of multiplex PCR in blood, urine and stool compared to results of SSS. On comparing the results of Multiplex PCR in blood and urine samples, out of 9 cases that tested positive in the urine samples, all of them tested positive in blood. Results in blood were positive in 82.4% of cases of those who were urine negative ($P < 0.001$). Similarly, all Multiplex PCR positive cases in stool samples were positive in blood samples and 86.4% of blood samples were positive in stool sample negative cases ($P < 0.001$) (**Table III**). Out of 9 cases who tested positive in urine samples, only 3 (33.3%) were stool positive; while stool samples were positive in 5.9% of cases where urine samples were negative ($P = 0.065$).

DISCUSSION

New diagnostic tools are required for early detection of *M.*

Table I Diagnostic Yield of RLEP PCR, Multiplex PCR and LAMP PCR for Urine, Stool and Blood Samples

	Urine n (%)	Stool n (%)	Blood n (%)
RLEP PCR Positive	7 (26.9)	2 (7.7)	21 (80.8)
Multiplex PCR Positive	9 (34.6)	4 (15.4)	22 (84.6)
LAMP PCR Positive	8 (30.8)	3 (11.5)	21 (80.8)

LAMP Loop mediated isothermal amplification, PCR Polymerase chain reaction, RLEP *Mycobacterium leprae*-specific repetitive element

leprae to ensure effective diagnosis and timely treatment of leprosy. PCR-based detection of *M. leprae* DNA in clinical samples has become increasingly important in the field of molecular diagnostics of leprosy especially in the tuberculoid spectrum of the disease where clinical manifestations are limited.

In the current study, we compared the diagnostic efficacy of multiplex PCR, RLEP PCR and LAMP PCR in the diagnosis of pediatric leprosy as an alternative to slit-skin smear examination. Out of the three PCR techniques, multiplex PCR offered best diagnostic yield in all the three samples including blood, urine and stool. Out of the three types of patient samples, multiplex PCR in blood had the best yield with 100% positivity in urine and stool-positive cases as well as 82.4% and 86.4% positivity in urine negative and stool negative cases ($P < 0.05$). Multiplex PCR in blood was positive in 83.3% of PB cases in comparison to SSS examination which was negative in all PB cases. Hence, blood multiplex PCR can be used for early diagnosis of leprosy, especially in PB cases. Compared to SSS examination, it is less invasive, less painful and free of human error. The results of multiplex PCR of urine and SSS examination are comparable and these tests can be used interchangeably.

Table II Comparative Performance of Multiplex PCR in Blood, Urine and Stool vs Slit-Skin Smear Examination

Tests	Total	SSS examination positive n (%)	SSS examination negative n (%)	P value (χ^2 test)	Percentage of agreement	Cohen's kappa
Blood						
Genetic material detected	23	7 (100)	16 (84.2)	< 0.001	38.46%	0.09
Genetic material not detected	3	0	3 (15.8)			
Urine						
Genetic material detected	9	6 (85.7)	3 (15.8)	0.001	84.62%	0.64
Genetic material not detected	17	1 (14.3)	16 (84.2)			
Stool						
Genetic material detected	4	3 (42.9)	1 (5.3)	0.018	80.76%	0.43
Genetic material not detected	22	4 (57.1)	18 (94.7)			

SSS Slit-skin smear

WHAT THIS STUDY ADDS?

- Multiplex PCR in blood is a much superior diagnostic test with a significantly higher positivity as compared to slit skin smear examination and can be used for diagnosis of both paucibacillary and multibacillary leprosy cases.

Table III Comparison of Yield of Multiplex PCR for *Mycobacterium leprae* in Blood, Urine and Stool Samples

Urine sample	Total	Blood sample	
		Positive n (%)	Negative n (%)
Positive	9	9 (100.0)	0
Negative	17	14 (82.4)	3 (17.6)
Total	26	23	3

$P < 0.001$, Percentage of agreement 46.15%, Cohen's kappa 0.13

Stool sample	Total	Blood sample	
		Positive n (%)	Negative n (%)
Positive	4	4 (100.0)	0
Negative	22	19 (86.4)	3 (13.6)
Total	26	23	3

$P < 0.001$, Percentage of agreement 26.92%, Cohen's kappa 0.05

Urine sample	Total	Stool sample	
		Positive n (%)	Negative n (%)
Positive	9	3 (33.3)	6 (66.7)
Negative	17	1 (5.9)	16 (94.1)
Total	26	4	22

$P < 0.065$, Percentage of agreement 73.07%, Cohen's kappa 0.32

Nearly a third patients tested positive by any of the PCR methods using urine samples in our study. Variation in *M. leprae* PCR positivity has been associated with the type of primers used, amplified fragment size, amplification techniques, and clinical specimens used [9]. The yield was best in blood samples by any of the PCR techniques. Previously, Wen et al used whole blood nested PCR amplification of *M. leprae* specific DNA for early diagnosis of leprosy [10].

The detection of *M. leprae* in urine and stool may be higher in patients on anti-leprosy treatment as small fragments of bacterial DNA may be found in urine samples as a result of DNA damage by antimicrobial therapy and its subsequent excretion [11]. Caleffi et al employed PCR-Pra for detection of *M. leprae* in urine samples of 73 leprosy patients (36 were under anti-leprosy multidrug therapy) and highlighted its diagnostic role in TT leprosy where the SSS is always negative [12].

We found a good agreement between the results of urine PCR and SSS examination with a Cohen's kappa

value of 0.64, indicating that the two tests can be used interchangeably. Multiplex PCR in blood detected all the SSS positive cases and 84.2% of SSS negative cases which is higher in comparison to urine and stool PCR. Moreover, blood multiplex PCR had poor agreement with SSS with a kappa value of 0.09 which infers that multiplex PCR offers superior diagnostic potential over SSS and can be used in diagnosis of leprosy in both SSS positive as well as negative cases.

The limitations of our study are the relatively small sample size, lack of control group and lack of comparison with tissue biopsy samples. Studies comparing the results of PCR on tissue biopsy specimen with results of PCR in urine, stool and blood samples with larger sample size are required. There is also the need to explore the role of PCR in screening the close contacts of patient for early diagnosis of leprosy.

Ethics clearance: No.: SNMC/IEC/THESIS/2023/151, dated Jan 09, 2023.

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