International Journal of Contemporary Pediatrics
Parvez M et al. Int J Contemp Pediatr. 2018 Nov;5(6):2059-2063
http://www.ijpediatrics.com
pISSN 2349-3283 | eISSN 2349-3291

DOI: http://dx.doi.org/10.18203/2349-3291.ijcp20184216

Original Research Article

Assessment of the diagnostic value of immunocytochemitsry and in situ hybridization in cytological specimen of childhood leprosy

Mohd. Parvez[1]*, R. Dayal[2], M. Natrajan[3], Raj Kamal[3]

[1]Department of Pediatrics, IIMSR, Lucknow, Uttar Pradesh, India
[2]Department of Pediatrics, SNMC, Agra, Uttar Pradesh, India
[3]Department of Clinical Medicine, JALMA, Agra, Uttar Pradesh, India

Received: 31 August 2018
Accepted: 12 September 2018

***Correspondence:**
Dr. Mohd. Parvez,
E-mail: njmsonline@gmail.com

ABSTRACT

Background: Hardly any studies have been done to study diagnostic value of immunocytochemistry and in situ hybridization in cytological specimens for the diagnosis of leprosy in children. The objective of this study is to assess the diagnostic value of immunocytochemistry and in situ hybridization in cytological specimens of leprosy patients. To compare these techniques with Z.N. staining.
Methods: This prospective study was carried out in 24 patients (≤18 years of age) of leprosy. Clinical examination of each patient was done and categorized according to IAL. After taking consent, three skin smears was taken, one for Z.N. staining and remaining two for immunocytochemistry and in situ hybridization respectively.
Results: Routine skin smear examination by ZN staining method confirmed the diagnosis in 2/24 (8.3%) cases and they belonged to BL category. Immunocytochemistry showed positivity in 4/7 (57.1%) in early leprosy (BT) and 82.3% (BB/BL) in late leprosy. Immunocytochemistry improved the diagnosis by 66.7%, and the results were statistically significant ($p<0.01$). In situ hybridization showed the positive results in 66.6% cases of early leprosy and 86.6% cases of late leprosy (BB/BL). In situ hybridization improved the diagnosis 72.6% in comparison to ZN staining and the results were statistically significant ($p<0.01$).
Conclusions: Immunocytochemistry and in situ hybridization enhance the diagnosis of leprosy when compared to routine skin smears stained by ZN staining. They are important diagnostic tools for definitive diagnosis in early as well as established cases of leprosy.

Keywords: Immunocytochemistry, In situ hybridization, Leprosy

INTRODUCTION

Leprosy is a chronic granulomatous disease caused by M. leprae. Leprosy is characterized by a long and variable incubation period and a chronic clinical course. Diagnosis of leprosy is essentially based on clinical feature. In an endemic country or area, an individual should be regarded as having leprosy if he or she shows one of the following cardinal signs.[1]

- Skin lesion consistent with leprosy and with definite sensory loss, with or
- Without thickened nerves
- Positive skin smears

Although the majority of cases can be diagnosed clinically yet alternative methods for diagnosis are required specially for early cases. Histopathological examination of skin biopsy can help in confirming

diagnosis in some of these early cases. Definite histopathological features are seen in only 35% of early cases.[2,3]

In the remaining cases the histology shows chronic inflammation referred to as nonspecific chronic inflammation (NSCI) and is common to many dermatoses. Children usually suffer from early form of the disease and it is important that diagnosis of leprosy is confirmed in these early cases.[4] Newer advanced methods like antigen detection in the lesion by immunostaining, amplification of DNA of M. leprae by PCR or demonstrating nucleic acid sequences specific to pathogen by in situ hybridization, help in confirming diagnosis of early cases.

Natrajan M, Katoch K et al studied the immunohistochemistry procedure on tissue biopsy which detect the mycobacterial antigen and exhibit 36.6% positivity.[5] Dayal R, Natrajan M et al demonstrating the nucleic acid sequence specific to *M. leprae* with help of in situ hybridization on tissue biopsy and observed 45.3% positivity.[6] However, tissue biopsy is a semi-invasive procedure and is difficult to do in pediatric age group.

Hardly any studies have been done to study diagnostic value of immunocytochemistry and in situ hybridization in cytological specimens for the diagnosis of leprosy in children. We conducted the study to evaluate the diagnostic value of immunocytochemistry and in situ hybridization in cytological specimens for the diagnosis of leprosy.

METHODS

The study was conducted at S. N. Medical College, Agra and National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra.

Twenty-four untreated leprosy cases, 18 years of age were included in the study. For evaluating the diagnostic value of immunocytochemistry and in situ hybridization over conventional Z N staining, both early as well as established cases were included in the study. These patients were thoroughly examined and classified clinically into BT (Borderline Tuberculoid), BB (Borderline Borderline) and BL (Borderline Lepromatous) types according to IAL classification.[7]

Clinical feature was recorded including number, size and location of lesions and loss of sensation. Any contiguous cutaneous nerve or peripheral nerve trunk enlargement was noted.

After taking consent three skin smears were prepared on silane coated slide from the active lesion. One slide was prepared for AFB detection by the Ziehl-Neelson's staining method and remaining two slides were fixed in 70% alcohol for 45 minutes and then stored for immunocytochemistry and in situ hybridization.

Immunocytochemistry

Stored slides were fixed with 4% paraformaldehyde and the following steps were performed:

Endogenous peroxidase blocking and blocking non-specific binding

After fixation, endogenous peroxidase was quenched with 0.3% hydrogen peroxide then blocking of non-specific binding to primary antibody was done with help of normal horse serum.

Incubation with primary and secondary antibody

The primary antigen detecting-antibody used was anti-M. bovis BCG (DAKO B0124). Binding of this antibody to mycobacterial antigens within the cytological specimens was detected by the sequential application of a biotinylated secondary antibody followed by horseradish peroxidase conjugated to Streptavidin-Biotin.

End product was visualized by using 3'3' diaminobenzidine as a chromogen and examine under microscope for yellow brown colour. Counterstain was done with Mayer's Hematoxylin and mounted with DPX

In-situ hybridization

This was performed in three major steps. After fixing with 4% paraformaldehyde. Premeabilization was done with 0.2N HCl, proteolysis with pepsin, post fixation with 4% paraformaldehyde and these steps was done to facilitate probe permeability into the cell.

Pre-hybridization with hybridization mixes minus probe was done at 42°C for 2 hrs. Denaturation prior to hybridization was done at 95°C for 6 minutes then immediately transferred into deep freezer for 3 minutes.

Hybridization

In this step solution containing digoxigenin labeled oligonucleotide probe targeting 16SrRNA of *M. leprae* and probe was used in final concentration of 1 µg/ml for hybridization procedure and added to each slide and incubated at 42ºC for overnight. Hybridization was done using hybridization chamber (Sigma)

Post hybridization washing and detection

Post-hybridization after cover slip removal was done with 2 × SSC followed by 1 × SSC. This was followed by an application of anti-digoxigenin antibody conjugated to AP (1:350) dilution. NBT/BCIP (1:50) dilution was added as a chromogen to obtained deep blue color in the end of procedure. Following development of colour, counter stain was done with 2% neutral red and finally mounted with DPX.

RESULTS

We studied 24 untreated patients, ≤ 18 years of age. Maximum number of patients were male (62.5%),6. Out of 24 cases,17/24 (70.8%) belonged to 12-18 years and remaining 5-11 years 7/24 (29.2%).

Table 1: Distribution of cases according to age and clinical category.

Age (years)	BT	BB	BL
5-11	3	3	1
12-18	4	11	2

Majority of patients 21/24 (87.5%) came in our OPD within 12 months of onset of illness. In present study, 75% presented with hypopigmented macular skin lesion in which most had ill-defined margins. Children having >4 skin lesions constituted 70.8% while remaining 29.2% had 1-3 skin lesions. Children having both skin lesions and nerve thickening constituted 79.2% while only skin lesions presented in 20.8%. Children had history of contact in 37.5% cases, out of which 29.5%cases were late leprosy (BB/BL) and 8.3% were early leprosy (BT). All patients with BT, BB were smear negative for AFB. Out of 24, 8.3% (2/24) were smear positive (Figure 1) and they belonged to BL category.

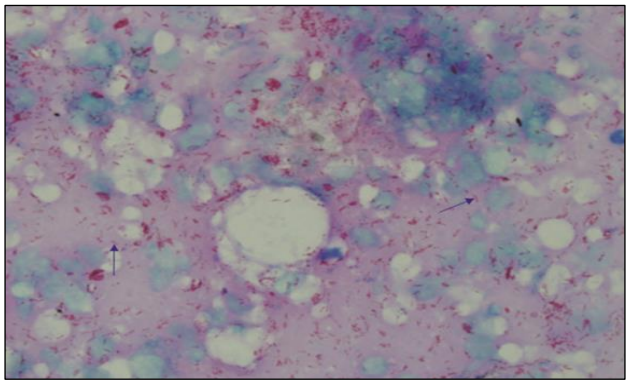

Figure 1: The acid-fast bacilli by ZN staining method.

Table 2: Correlation of results of immunocytochemistry with ZN staining under study.

Clinical type	No. tested	ICC (+ve signal)		ZN staining (+ve signal)		Enhancement in diagnosis %
		No.	%	No.	%	
BT	7	4/7	57.1	-	-	57.10
BB	14	11/14	78.50	-	-	78.50
BL	3	3/3	100.0	2/3	66.6	100.00
Total	24	18	75.0	2	8.3	66.7

BT: Borderline tuberculoid; BB: Borderline; BL: Borderline lepromatous; χ^2: 21.94; P = <0.01

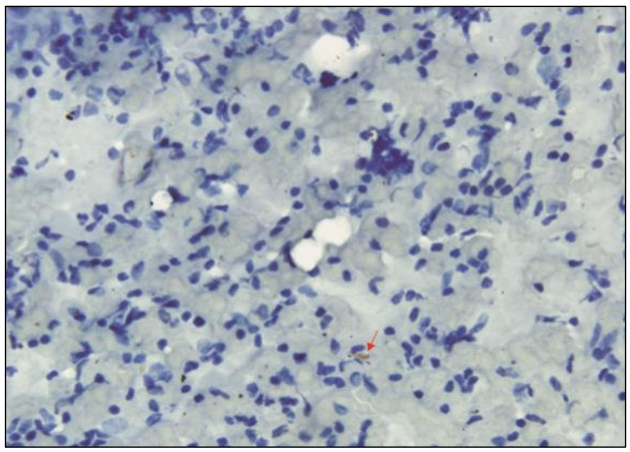

Figure 2: Immunocytochemistry on cell smear of BT case showing positive signal as a yellow, brown end product (magnification 300x).

Immunocytochemistry was done in all 24 cases targeting Mycobaterial antigen (Figure 2 and 3) using anti *M. bovis*, BCG (DAKO B0124). We observed positive results in 4/7(57.1%) of early leprosy (BT) and 14/17 (82.3%) in late leprosy (BB/BL). Over all immunocytochemistry gave positive results in 18/24 (75%) cases. Immunocytochemistry improved the diagnosis by 66.7% over ZN staining and the results were found to be statistically significant (p<0.01).

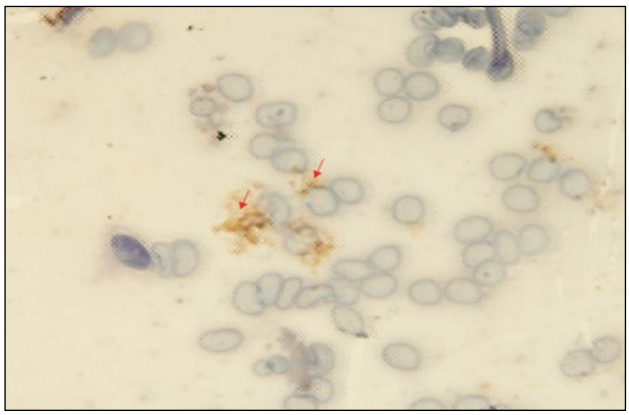

Figure 3: Immunocytochemistry on cell smear of BL case showing positive signal as a yellow, brown end product (magnification 200x).

In situ hybridization was done using oligonucleotide probe targeting 16SrRNA of *M. leprae* (Figure 4 and 5).

In situ hybridization was done on 24 cases. Three samples were washed out during the procedure.

Table 3: Correlation of results of in-situ hybridization with ZN staining under study.

Clinical type	No. tested	Zn staining (+ve signal)		In situ hybridization (+ve cases)		Enhancement in diagnosis %
		No.	%	No.	%	
BT	6	-	-	4/6	66.6	66.6
BB	15	-	-	10/12	83.3	83.3
BL	3	2/3	66.6	3/3	100.0	100.0
Total	24	2/24	8.3	17/21	80.1	72.6

BT: Borderline tuberculoid; BB: Borderline, BL: Borderline lepromatous; $\chi^2 = 24.21$; $p = < 0.01$

We observed 21 slides and got positive results in 4/6 (66.6%) in early leprosy and 13/15 (86.6) in late leprosy (BB/BL). Overall positivity was 17/21 (80.9%) which improved the diagnosis by 72.6%.

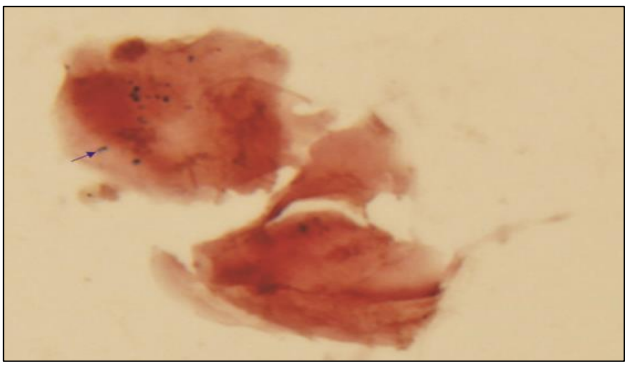

Figure 4: In situ hybridization on cell smear of BT case showing positive signal as a deep blue colour (magnification 300x).

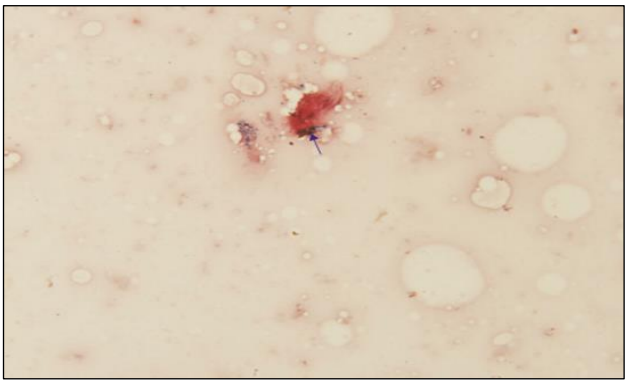

Figure 5: In situ hybridization on cell smear of BL case showing positive signal as a deep blue colour (magnification 200x).

When results of in situ hybridization were compared to ZN staining, they were found to be statistically significant ($p<0.01$).

DISCUSSION

This study comprised of 24 cases. 62.5% were male. Out of 24 cases, 21/24 (87.5%) came to our OPD within 12 months of onset of illness. The results were comparable 6-12 with other authors reported earlier.

In present study 75% children had hypopigmented and macular lesion. Children having >4 skin lesions constituted 70.8% while remaining had 1-3 skin lesions. In present study 37.5% had contact history, in which 29.2% belonged BB/BL category while only 8.3%

belonged BT category. These results were comparable with other studies by other authors.[8,9] All cases of BB and BT were skin smear negative. 2/24(8.3%) cases were skin smear and they belonged to BL category.

In present study immunocytochemistry was done on 24 sample. Out of 24 cases, we observed 18(75%) were positive. Immunocytochemistry diagnosed early leprosy (BT) in 4/7(57.1%) cases and late leprosy (BB/BL) in 82.3% (14/17) cases. Immunohistochemistry improved diagnosis by 66.7% in comparison to ZN staining. This enhancement in diagnosis was statistically significant ($p<0.01$).

In situ hybridization was done on 24 samples, out of which 3 samples were washed out. Out of 21 samples examined, 80.9% (17/21) were positive. In situ hybridization diagnosed early leprosy in 4/6(66.6%) and late leprosy (BB/BL) in 13/15 (86.3%). In situ hybridization improved diagnosis by 72.16% in comparison to ZN staining which was statistically significant ($p<0.01$).

Immunocytochemistry and in situ hybridization may not be routinely required to confirm clinical diagnosis. However, it may have wider application in doubtful cases and additionally as a research tool to study early diagnosis.[10-12]

In present study, 75% positivity in immunocytochemistry and 80.9% positivity in in situ hybridization was found in cytological specimens. Immunocytochemistry and in situ hybridization can diagnose early leprosy (BT) in 57% and 66.6% cases respectively. In situ hybridization improved diagnosis by 5.1% in comparison to immunocytochemistry, but it is more complex and a time-consuming procedure.

Thus, immunocytochemistry and in situ hybridization had excellent results for the diagnosis of early as well as late cases of leprosy. However, these methods need further evaluation on a larger sample size.

CONCLUSION

Immunocytochemistry and in situ hybridization enhance the diagnosis of leprosy when compared to routine skin smears stained by ZN staining. They are important diagnostic tools for definitive diagnosis in early as well as established cases of leprosy.

Funding: No funding sources
Conflict of interest: None declared
Ethical approval: The study was approved by the Institutional Ethics Committee

REFERENCES

1. WHO. Diagnosis of leprosy. 2011. Available at www.who.int/lep/diagnosis/en/index.
2. Fine PE, Job CK, McDougall AC, Meyers WM, Ponnighaus JM. Comparability among histopathologists in the diagnosis and classification of lesions suspected of leprosy in Malawi. Int J Lepr. 1986;54:614-25.
3. Ramu G, Karthikeyan S, Balakrishanan S. Histological and Immunological correlation of suspected leprosy patients. Indian J Lepr. 1996;68: 153.
4. Wang XM, Chan SY, Ran SP. Immunohistopathology in the diagnosis of early leprosy. Int J Lepr. 2000;68:426-9.
5. Natrajan M, Katoch K, Katoch VM. Histology and immuno-histology of lesions clinically suspicious of leprosy. Acta leprologica. 1999;11(3):93-8.
6. Dayal R, Agarwal M, Natrajan M, Katoch VM, Katoch K, Singh K. PCR and in-situ hybridization for diagnosis of leprosy. Indian J Pediatr. 2007;74(7):645-8.
7. Dharmendra. Classification of leprosy. In: Hastings RC, Convit J, Eds. Leprosy. Churchill Livingstone, Edinburgh; 1985:88-99.
8. Dayal R, Gupta R, Mathur PP, Dhir GG, Katoch VM, Katoch K. Study of gene probes in childhood leprosy. Ind J Pediatr.1997;65:99-105.
9. Dave MS, Agarwal SK. Prevalence of leprosy in children of Leprosy patients. Lepr India. 1984;56:615-21.
10. Dayal R, Hashmi NA, Mathur PP, Prasad R. Leprosy in children. Indian Pediatr. 1990;27:170-80.
11. Dayal R, Agrawal PK, Kalra K, Bhardwaj VP, Katoch VM, Katoch K. Diagnostic value of gene probes and its correlation with clinical profile of leprosy in Children. Indian Pediatr. 1994;31:1521-7.
12. Natrajan M, Katoch K, Katoch VM. Increase in the sensitivity of immunohistochemical procedure by end product amplification demonstrated on tissue specimens of clinically suspect leprosy. Indian J Lepr. 2003;76:64-5.

Cite this article as: Parvez M, Dayal R, Natrajan M, Kamal R. Assesment of the diagnostic value of immunocytochemitsry and in situ hybridization in cytological specimen of childhood leprosy. Int J Contemp Pediatr 2018;5:2059-63.