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Original Article

Evaluation of the diagnostic value of immunocytochemistry and *in situ* hybridization in the pediatric leprosy

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Leprosy is characterized by a long and variable incubation period and a chronic clinical course. Diagnosis of leprosy is essentially based on clinical features. Although the majority of cases can be diagnosed clinically yet alternative methods for diagnosis are required especially for early cases. Immunocytochemistry and *in situ* hybridization can be a valuable tool for diagnosis for early cases. The present study is aimed to assess the diagnostic value of immunocytochemistry and *in situ* hybridization in cytological specimens and to compare these techniques with Z.N. staining. This prospective study was carried out in 26 patients below 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. Routine skin smear examination by Z.N. staining method confirmed the diagnosis in 4/26 (15.83%) and these belonged to BB, BL category. Immunocytochemistry showed positivity in 10/15 (66.6%) in BT and 72.7% in BB/BL leprosy. Immunocytochemistry improved the diagnosis by 53.85%, and the results were statistically significant ($p<0.01$). *In situ* hybridization showed the positive results in 80% cases of BT leprosy and 90.9% cases of BB/BL leprosy. *In situ* hybridization improved the diagnosis by 70% in comparison to ZN staining and the results were statistically significant ($p<0.01$). This study supports that immunocytochemistry and *in situ* hybridization enhance the diagnosis of leprosy when compared to routine skin smears stained by Z.N staining. They are important diagnostic tools for definitive diagnosis in early as well as established cases of leprosy.

Key words : Leprosy, Immunocytochemistry, *In situ* hybridization

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 features. It is diagnosed by the presence of at least two of the three cardinal signs enumerated below or the last one independently

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(Dharmendra and Chatterjee 1978). (i) Hypo-pigmented patch with Loss or impairment of cutaneous sensation, (ii) Thickening of nerves, (iii) Presence of acid fast bacilli in the skin or nasal 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 (Fine PEM et al 1986, Ramu G at al 1996). In the remaining cases the histology shows chronic inflammation referred to as nonspecific chronic inflammation (NSCI) (Xiao Man Wang et al 2000) 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. 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 (Natrajan M et al 1999) studied the immunohistochemistry procedure on tissue biopsy which detect the mycobacterial antigen and exhibit 36.6% positivity. Dayal R, Natrajan M et al, (Dayal R, Natrajan M et al 2007) demonstrated the nucleic acid sequence specific to *M. leprae* with help of *in situ* hybridization on tissue biopsy and observed 45.3% positivity. However, tissue biopsy is a semi-invasive procedure and is difficult to do in pediatric age group.

However, hardly any studies have been done to study diagnosis 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.

Material and Methods

The study was conducted at SN Medical College, Agra and National JALMA Institute of Leprosy and Other Mycobacterial Diseases, Agra. Twenty six untreated leprosy cases, below 18 year of age were included in the study. For evaluating the diagnostic value of immunocytochemistry and in situ hybridization over conventional ZN staining, both BT as well as BB/BL 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 (Classification of Leprosy 1982).[7] Clinical features were 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 saline 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 immuno-cytochemistry and in situ hybridization.

Immunohistochemistry

Stored slides were fixed with 4% paraform-aldehyde and the following steps were per-formed:

1. Endogenous Peroxidase blocking and block-ing 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.

2. Incubation with Primary and secondary Antibody: The primary antigen detecting-antibody used was anti-*M. bovis* BCG (DAKO B0124). Binding of this antibody to myco-bacterial antigens within the cytological specimens was detected by the sequential application of a biotinylated secondary antibody followed by horseradish peroxidase conjugated to Streptavidin-Biotin.
3. End product was visualized by using 3'3' diaminbenzidine as a chromogen and exam-ine under microscope for yellow brown colour (Fig 1).
4. Counterstain was done with Mayer's Hema-toxylin and mounted with DPX.

***In-situ* Hybridization**

1. 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 step was done to facilitate probe permeability into the cell. Pre-hybridization with hybridization mix 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.
2. **Hybridization:** In this step solution con-taining 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 42°C for over night. Hybridization was done using hybridization chamber(Sigma).
3. **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 antidigoxigenin antibody conjugated to AP

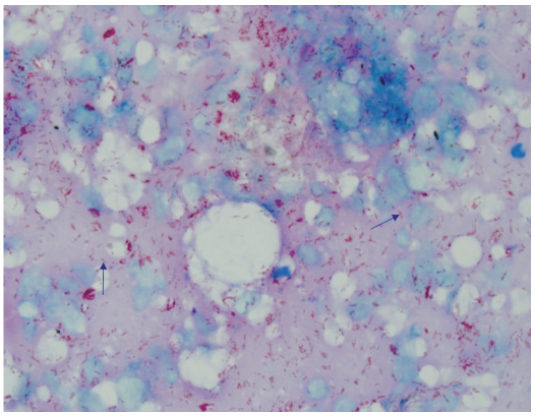

Fig 1 : Showing the Acid Fast bacilli by ZN method (Magnification-300x)

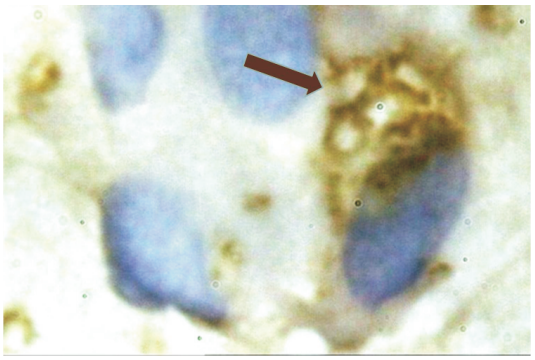

Fig 2 : Immunocytochemistry on cell smears of BT Case showing positive signals as a yellow browne end product (original Magnification-400x)

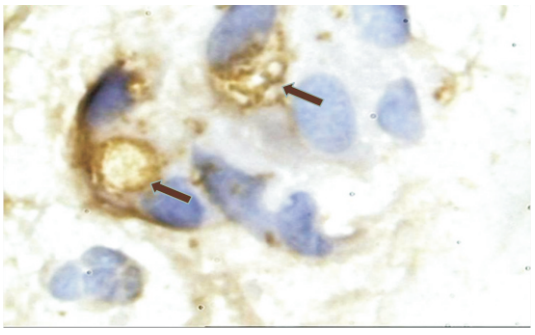

Fig 3 : Immunocytochemistry on cell smears of BL Case showing positive signals as a yellow browne end product (original Magnification-400x)

(1:350) dilution. NBT/BCIP (1:50) dilution was added as a chromogen to obtained deep blue colour 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 26 untreated patients, < 18 years of age. Maximum number of patients were male (75%), Out of 26 cases, 16/26 (61.53%) belonged to 11-16 years and remaining were 5-10 years 10/26(38.46%).

Table 1 : Distribution of Cases According to Age and Clinical Category

Age groups (years)	Clinical category BT	BB	BL	Total (%)
5-10	6	3	1	10/26 (38.46%)
11-18	9	5	2	16/26 (61.53%)
Total (%)	15/26 (57.69%)	8/26 (30.76%)	3/26 (11.53%)	26 (100%)

Table 2 : Association of Results if *In-situ* Hybridization with ZN staining under study

Clinical type	Numbers tested	*In situ* hybridization PCR (+ve signal) Numbers	%	ZN staining (+ve signal) Numbers	%	Enhancement in diagnosis %
BT	15	12/15	80%	-	-	80.00%
BB	08	7/8	87.50%	2/8	25%	62.50%
BL	3	3/3	100.0	2/3	66.6%	33.40%
Total	26	22/26	84.61%	4/26	15.38%	70%

BT= Borderline tuberculoid; BB = Borderline, BL= Borderline lepromatous

Chi^2 = 21.94

p = < 0.01

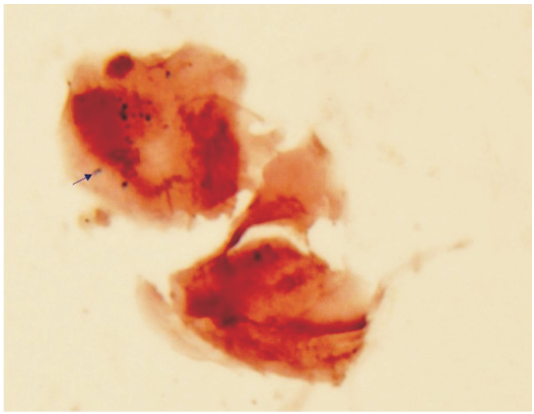

Fig 4 : *In situ* hybridization of cell smear of a BT cases showing positive signals as deep blue colour (Orig –Magnification 300x)

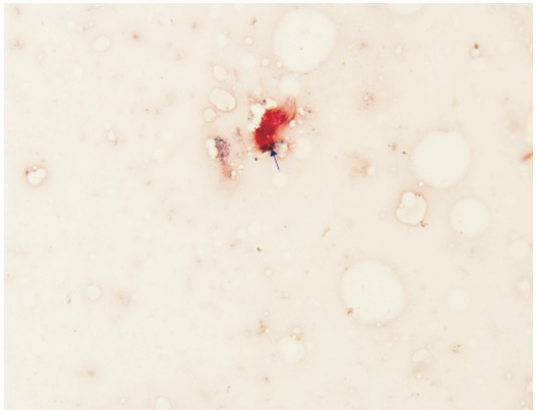

Fig 5 : *In situ* hybridization of cell smear of a BL cases showing positive signals as deep blue colour (Orig –Magnification 200x)

Table 3 : Association of Results of Immuno Cyto Chemistry with ZN staining under study

Clinical type	Numbers tested	ZN staining (+ve signal)		ICC (+ve signal)		Enhancement in diagnosis
		Numbers	%	Numbers	%	%
BT	15	-	-	10/15	66.6	66.6
BB	8	2/8	25%	5/8	62.5%	37.50
BL	3	2/3	66.6%	3/3	100.0	33.00
Total	26	4/26	15.38	18/26	69.23	53.85

BT= Borderline tuberculoid; BB = Borderline, BL= Borderline lepromatous

Chi^2 = 24.21

p = < 0.01

Majority of patients 23/26 (88.46%) came in our OPD within 12 months of onset of illness. In our study, Majority 75% of the cases were male. Majority (64%) of the patients had history of contact with a leprosy patients with in the family. Most of the patients (60%) had hypopigmented lesions only 10% had erythmatous lesions. 60% had well defined margins and 40% had ill defined margins. Nerve thickning was observed in 38% of cases. As age advanced the disease moved from tuberculoid end of the spectrum towards lepromatous end. 68% of cases were unimmunized. Skin smears were negative for AFB in 84% of the cases 16% cases were positive for AFB. Children having >4 skin lesions constituted 70.8%, while remaining 29.2% had 1-3 skin lesions. were (BB/BL) leprosy and 57.69% were (BT) leprosy.

Immunocytochemistry was done in all 26 cases targeting Mycobaterial antigen using anti *M. bovis*, BCG (DAKO B0124). We observed positive results in 10/15 (66.6%) of BT and 8/11 (72.72%) in BB/BL leprosy. Over all, immunocytochemistry gave positive results in 18/26 (69.9%) cases. Immunocytochemistry improved the diagnosis by 53.85% over Z.N. staining and the results were found to be statistically significant (p<0.01).

In situ hybridization was done using oligonucleotide probe targeting 16SrRNA of *M. leprae. In situ* hybridization was done on 26 cases. On applying in situ hybridizationon slit skin smears it was observed that enhancement of diagnostic yields was 80% in cases of BT category of pediatric leprosy. Enhancement of diagnostic yields was 62.5%, and 33.5% in cases of BB/ BL category of pediatric leprosy respectably. Overall enhancement of diagnostic yields was 70% in Borderline cases of pediatric leprosy.

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

On applying Immunocytochemistry on slit skin smears it was observed that enhancement of diagnostic yields was 66% in cases of BT category of pediatric leprosy. Enhancement of diagnostic yields was 37.5%, and 33.4% in cases of BB/BL category of pediatric leprosy respectably. Overall enhancement of diagnostic yields was 53.85% in cases Borderline cases of pediatric leprosy.

Discussion

This study comprised of 26 cases. 75% were male. Out of 26 cases, 23/26 (88.46%) came to our OPD with in 12 months of onset of illness. The results were comparable (Dayal R, Agarwal M et al 2007, Classification of Leprosy 1982, Dayal R, Gupta R et al 1997, Dave MS and Agarwal SK et al 1984, Dayal R, Hashmi NA et al 1990, Dayal R, Agrawal PK 1994) with other authors reported earlier.

In our study 75% children had hypopigmented and macular lesion. Children having >4 skin

lesions constituted 70.8% while remaining had 1-3 skin lesions. In our study majority of children 64% had contact history, in which 40.9% belonged BB/BL category while 23.1% belonged BT category. These results were comparable with other studies by other authors (Dayal R Gupta R et al 1997, Dave MS and Agarwal SK 1984). All cases of BT were skin smear negative. 4/26 (15.83%) cases were skin smear and they belonged to BB, BL category. In our study, immunocytochemistry was done on 26 samples. Out of 26 cases, we observed 18/26 (69.23%) were positive. Immunocytochemistry diagnosed BT leprosy in 10/15 (66.6%) cases and in BB/BL leprosy 72.72% (8/11) cases. Immunohistochemistry improved diagnosis by 53.85% in comparison to ZN staining. This enhancement in diagnosis was statistically significant ($p<0.01$). *In situ* hybridization was done on 26 samples, Out of 26 samples examined, 84.61% (22/26) were positive. *In situ* hybridization diagnosed BT leprosy in 2/15 (80%) and BB/BL leprosy in 10/11 (90.9%). *In situ* hybridization improved diagnosis by 70% 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. In our study, 69.23% positivity in immunocytochemistry and 84.61% positivity in *in situ* hybridization was found in cytological specimens. Immunocytochemistry and *in situ* hybridization can diagnose early leprosy (BT) in 66.66% and 80% cases respectively. *In situ* hybridization improved diagnosis by 13.34% 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(BT) as well as established cases (BB/BL) of leprosy. However, these methods need further evaluation on a larger sample size.

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