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A Comparative Evaluation of ELISA Employing Antigen 85C with Skin Slit Smear in Diagnosis of Leprosy in Children

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ABSTRACT

Leprosy, caused by Mycobacterium leprae, presents with hypopigmented patches and hardening of nerves. Early diagnosis is crucial, especially in children. Current diagnostic methods include histopathological examination, polymerase chain reaction (PCR) and immunostaining, but these are limited and expensive. This study aims to compare the diagnostic value of skin slit smear for acid-fast bacilli (AFB) and Enzyme linked immunosorbent assay (ELISA) based detection of antibodies against Antigen 85C (Ag85C) for diagnosis of leprosy in paediatric population. The present prospective study included 30 children, below 16 years age with leprosy lesions and no prior history of anti-lepromatous treatment. After institutional ethical approval, the relevant clinical history and demographic details were recorded. Skin smears were collected and examined for AFB. ELISA was used to detect Ag85C antibodies in the serum of all patients. The 30 pediatric leprosy cases, included were of predominantly Borderline tuberculoid (BT) type. Male-to-female ratio was 1.5:1. Most cases had skin lesions and nerve trunk thickening. Ulnar nerve thickening was common in nerve involvement cases. AFB detection by skin slit smears was only 26.66% (08/30 cases), while ELISA based detection of Ag85C antibodies was positive in 83.33% (25/30 cases). ELISA for Ag85C antibodies showed 100% specificity in diagnosing leprosy. Moreover skin slit smear was universally negative in all cases of tuberculoid (TT) and borderline tuberculoid (BT), whereas ELISA was positive in 40% cases of TT and 86.6% cases of BT as well. Thus, ELISA had higher detection rate than AFB smear, especially in TT and BT type of leprosy. Overall, ELISA outperformed AFB smear in diagnosing leprosy cases ($p<0.0009$). The diagnostic potential of ELISA based antibody test, targeted against Ag85C in serum samples is a sensitive tool for diagnosis of early leprosy cases with negative AFB slit skin smears and no feasible skin biopsy. Larger studies are needed for further validation of results of present study.

INTRODUCTION

Leprosy is a chronic infective disease caused by *Mycobacterium leprae* and is clinically characterized by an extended and erratic incubation period. The detection of leprosy is based on the presence of vital clinical features like formation of hypopigmented patches with no or decline in the cutaneous sensation, hardening of nerves and demonstration of positive results for the presence of acid fast bacilli in the skin or nasal samples. Albeit, most of the cases are diagnosed clinically, still accurate and reliable methods are required for the detection of disease at an early stage. Histopathological analysis of skin biopsy may confirm the leprosy in budding stage, but they are limited. Further, histopathological analysis shows higher detection rate for certain types of leprosy like Borderline-borderline leprosy (BB), Borderline lepromatous leprosy (BL) and Lepromatous leprosy (LL)[1].

Diagnosis of leprosy at an early stage is highly imperative, especially in children since they are highly susceptible at an early period[2]. Novel molecular methods which encompasses antigen detection in the lesion by immunostaining, polymerase chain reaction (PCR) amplification of *M. leprae* DNA and *In-situ* hybridization technique which synthesis specific DNA to the pathogen of our interest[3,4]. Despite early diagnosis, these methods impose high economic burden with minimal sensitivity. Recently, no clinical study has been performed to evaluate the diagnostic value of serology in childhood leprosy. With this backdrop, we conducted this study to evaluate the diagnostic value of ELISA based detection of antibody against Ag85C in paediatric leprosy.

MATERIALS AND METHODS

Thirty children attending the outpatient department of National Jalma Institute of Leprosy and Other Mycobacterial Disease, Agra and Department of Pediatrics, S.N. Medical College, Agra were prospectively enrolled for the study after proper informed consent. The study was ethically approved by Institutional Ethics Committee. The demographic details and detailed clinical history of patients were recorded like, age, gender, history of contact with leprosy cases, site and duration of lesion, charting and description of skin lesion and nerve involvement if any. The cases were classified according to Ridley and Jopling Classification as polar tuberculoid (TT), borderline tuberculoid (BT), mid-borderline (BB), borderline lepromatous (BL) and polar lepromatous (LL) leprosy.

Inclusion criteria: Patient below or equal to 16 years of age with lesions of leprosy and who did not have history of anti-lepromatous treatment were included in the study.

Microscopic examination of skin slit smears: Skin slit smears were taken from all suspects and leprosy cases to identify the presence of acid fast bacilli (AFB). The smears were taken using a standard procedure and were stained by modified Ziehl Neelsen staining before subjecting them to oil immersion light microscopy and were classified bacteriologically on Ridley's scale.

Ag85C antibody detection by ELISA: About 100 µL of the Ag85 complex, Ag85C (25 ng mL^{-1}) was coated on a 96-well ELISA plate in 0.05 M carbonate bicarbonate buffer, pH9.6. The working dilution of each antigen was standardized using checker board titration. After overnight incubation at 37°C, the contents of the wells were aspirated and the plate was washed 3 times with 150 mM PBS, pH 7.4. The plate was blocked with 2% BSA in PBS (200 µL $well^{-1}$) for one hour. After washing with PBST (with 0.05% Tween-20), serum diluted 1:200 in assay diluent (1% BSA in PBS with 0.05% Tween 20) was added in duplicate wells and incubated at 37°C for 2 hrs. Anti-human IgG peroxidase-conjugated antibody (Sigma, St. Louis, MO, USA) diluted 1:10000 in assay diluents was added to each well. After incubation for 1 hrs, 100 µL of substrate solution [orthophenylenediamine (OPD) tablet of 5 mg dissolved in 10 mL of distilled water and 50 µl H_2O_2] was added to each well and kept at room temperature for 20 min in the dark. The reaction was stopped by adding 50 µL of 7% H_2SO_4 (stop solution) to each well and the absorbance was measured at 492 nm using a Spectramax-M2 Reader (Molecular Devices, Sunnyvale, CA, USA). All the experiments were carried out twice.

Statistical analysis: The data were analyzed using statistical package for social sciences (SPSS) software version 20.0. Categorical variables were analyzed by Chi-square (univariate) test. The Fischer exact test was applied to assess the level of significance, wherever the values were 5 or less. A p-value of <0.05 was considered as statistically significant.

RESULTS

In the present study, the male to female ratio was found to be 1.5:1. Most patients were between the age of 12-15 years. Twenty one cases (70.0%) were between 11-16 years of age and 9 cases (30.0 %) were < 10 years of age. Majority of patients were diagnosed with BT leprosy type (15/30 cases, 50%). The results including demographic characteristics and clinical features are summarized in Table 1 and exact number

Table 1: Demographic and clinical characteristics of paediatric leprosy patients

Demographic and clinical features	No of cases/Total cases	Percentage
Age		
11-16 years	21/30	70.0
<10 years	09/30	30.0
Gender		
Males	18/30	60.0
Females	12/30	40.0
Contact with leprosy in family	08/30	26.6
Skin lesions		
Single lesion	06/30	20.0
2-4 lesions	13/30	43.3
>5 lesions	11/30	36.6
Both skin lesions and nerve thickening	22/30	73.3
Skin lesions with only ulner nerve thickening	10/22	45.4
Skin lesions with both ulner and popliteal nerve thickening	12/22	54.5

Table 2: Comparison of results of skin slit smear for AFB and ELISA based detection of Ag85C in sera in various types of paediatric leprosy cases

Leprosy types	No. of AFB positive cases (percentage)	No of cases detected by ELISA based Ag85C (percentage)*
TT (n = 05)	0	02 (40%)
BT (n = 15)	0	13 (86.6%)
BB (n = 08)	06 (75.0%)	08 (100%)
BL (n = 01)	01 (100%)	01 (100%)
LL (n = 01)	01 (100%)	01 (100%)
Total (N = 30)	08 (26.66%)	25 (83.33%)

*All 30 age matched controls were negative by ELISA, $\chi^2 = 17.24$, p<0.0009, df = 1, Chi Square test shows that p-value is highly significantSystem Generated list

of cases in each category of leprosy type with results of skin slit smear for AFB and ELISA based detection of antibodies against Ag85C are mentioned in Table 2.

Slit Skin smear detection for acid fast bacilli (AFB): The results of the study indicate that special stain for AFB was negative in most of the patients and the overall positivity of slit skin smears for AFB was only 26.6%. Among the leprosy types, AFB positivity was seen in all cases of BL and LL type and in 6 out of 8 (75.0 %) cases of BB type. However, in TT and BT types of leprosy all the cases were found to be negative for AFB. (Table 2).

ELISA based detection of antibody against Ag85C: ELISA against Ag85C detected 25 out of 30 patients and thus confirmed the diagnosis in 83.33% cases in the study. Similarly, controls were taken from inpatients of department of pediatrics who did not have leprosy and all the 30 age matched control samples were negative by ELISA (Table 2).

Correlation of skin slit smear for AFB and ELISA using Ag85C: Of total 30 patients, skin slit smear was positive for AFB in only 8 cases (26.66%) while ELISA based detection of Ag85C was positive in 25 cases (83.33%). Thus, the outcome of the results by ELISA method showed significantly higher diagnostic value than slit smear examination for AFB in diagnosing leprosy. (p<0.0009) (Table 2).

DISCUSSION

Out of thirty cases included in the present study (Table 2), 15 cases (50%) were of BT type, 5 cases of TT, 1 case of BL and LL each and rest 8 cases were of BB type of leprosy. Thus the present study includes predominantly early forms (TT, BT, BB) of leprosy, which are often difficult yet crucial to diagnose at an early stage.

In this study, most of the cases were in adolescent age group (21 patients between 11-16 years age) and this may be explained by the fact that leprosy has long incubation period and needs prolonged exposure. Preschool children were less as compared to school going age group and this observation is comparable to study done by Ganapati *et al*.[5] who reported leprosy is more common in school age group as compared to preschool children.

Further it was observed that male cases (60%) were more as compared to female (40%) cases and M:F (ratio) was 1.5:1. This observation was comparable to the results of the study done by Dayal *et al*.[6] who reported male preponderance in their studies. This is a small study sample and therefore further comments cannot be made on sex ratio of patients included in this study.

We also noted that overall 26.6% of cases had leprosy in family of which 3 cases were of BT type and 5 cases were BB/BL/LL type of leprosy. This observation is similar to study done by Dayal *et al*.[7] who reported 31.8 % cases having family history (more in mutibacillary type).

In this study there were 6 cases having single lesion. Maximum number of cases (13 cases, 43.3%) had 2-4 skin lesions and remaining 11 cases had >5 skin lesions (36.6%). We also found that 22 cases (73.3 %) had both skin lesions and nerve trunk thickening and only 8 cases (26.6 %) had skin lesions without nerve

trunk thickening. There were no cases having only nerve involvement in this study. Further we noted that, of 22 cases having nerve involvement, 10/22 (45.45%) cases had only ulnar nerve thickening and 12/22 (54.55%) cases had both ulnar and lateral popliteal nerve thickening. There was no child with pure neuritic leprosy . The results are comparable to the finding of Dayal *et al*.[7].

All the cases of TT and BT type of leprosy were smear negative for AFB. Skin smear were positive for AFB in 8/30 cases (26.66%) of BB, BL and LL type of leprosy.

ELISA based antibody against Ag85C positivity was observed in 25/30 cases (83.33%) of leprosy [13/15 cases of BT type, 6/ 8 cases of BB type, 2/5 cases of TT, 1/1 case of BL and 1/1 cases of LL type of leprosy]. All age matched (30) controls were negative (100% specificity). There were no cases which were positive for skin smear for AFB and ELISA was negative. In this study low rate of detection was seen in BT type of leprosy as compared to other types . We have used *M. tuberculosis* Ag85C hence lesser positivity was observed, taking Ag85C from *M. leprae* could increase the sensitivity further. Overall ELISA based antibody detection has good sensitivity and specificity in diagnosing leprosy cases and observations are statistically significant ($p<0.0009$). Earlier antibody based ELISA was used for diagnosis of pediatric TB cases and it showed good diagnostic potential. Our results are similar to those findings[8].

The results of ELISA based antibody detection against Ag85C on serum specimens in this study are highly significant. In this study when we compared the results of slit skin smear for AFB and Ag85C based ELISA, it was found that Ag85C based ELISA was positive in 17 cases, which were negative by skin smear for AFB, 8 cases were positive by both methods and no cases were negative by Ag85C based ELISA but were positive by skin smear for AFB. This shows that Ag85C based ELISA enhances diagnosis as compared to slit skin smear. This observation is statistically significant ($p<0.0009$)[9-17].

CONCLUSION

Early cases of leprosy pose challenges in diagnosis, especially in areas with lack of histopathological examination facility. Moreover the polar tuberculoid end of leprosy spectrum and borderline cases (TT, BT and BB) often require more than one sensitive diagnostic modalities for confirmation. The present study shows that novel technique of ELISA based Ag85C detection on serum samples is a highly sensitive and specific tool used in confirming diagnosis of early leprosy cases where slit skin smears for AFB are negative and skin biopsy is not feasible. Furthermore larger studies with more number of subjects are needed to generalize the results of present study.

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